

1      **Chimeric binding peptide library screening method**

2  
3      The present invention relates generally to methods for  
4      screening nucleotide libraries for sequences that  
5      encode peptides of interest.

6  
7      Isolating an unknown gene which encodes a desired  
8      peptide from a recombinant DNA library can be a  
9      difficult task. The use of hybridisation probes may  
10     facilitate the process, but their use is generally  
11     dependent on knowing at least a portion of the sequence  
12     of the gene which encodes the protein. When the  
13     sequence is not known, DNA libraries can be expressed  
14     in an expression vector, and antibodies have been used  
15     to screen for plaques or colonies displaying the  
16     desired protein antigen. This procedure has been useful  
17     in screening small libraries, but rarely occurring  
18     sequences which are represented in less than about 1 in  
19      $10^5$  clones (as is the case with rarely occurring cDNA  
20     molecules or synthetic peptides) can be easily missed,  
21     making screening libraries larger than  $10^6$  clones at  
22     best laborious and difficult. Methods designed to  
23     address the isolation of rarely occurring sequences by  
24     screening libraries of  $10^6$  clones have been developed  
25     and include phage display methods and LacI fusion phage  
26     display, discussed in more detail below.

27  
28     Phage display methods. Members of DNA libraries which  
29     are fused to the N-terminal end of filamentous  
30     bacteriophage pIII and pVIII coat proteins have been  
31     expressed from an expression vector resulting in the

1 display of foreign peptides on the surface of the phage  
2 particle with the DNA encoding the fusion protein  
3 packaged in the phage particle (Smith G. P., 1985,  
4 Science 228: 1315-1317). The expression vector can be  
5 the bacteriophage genome itself, or a phagemid vector,  
6 into which a bacteriophage coat protein has been  
7 cloned. In the latter case, the host bacterium,  
8 containing the phagemid vector, must be co-infected  
9 with autonomously replicating bacteriophage, termed  
10 helper phage, to provide the full complement of  
11 proteins necessary to produce mature phage particles.  
12 The helper phage normally has a genetic defect in the  
13 origin of replication which results in the preferential  
14 packaging of the phagemid genome. Expression of the  
15 fusion protein following helper phage infection, allows  
16 incorporation of both fusion protein and wild type coat  
17 protein into the phage particle during assembly.  
18 Libraries of fusion proteins incorporated into phage,  
19 can then be selected for binding members against  
20 targets of interest (ligands). Bound phage can then be  
21 allowed to reinfect *Escherichia coli* (*E. coli*) bacteria  
22 and then amplified and the selection repeated,  
23 resulting in the enrichment of binding members  
24 (Parmley, S. F., & Smith, G. P. 1988., Gene 73: 305-  
25 318; Barrett R. W. et al., 1992, Analytical  
26 Biochemistry 204: 357-364 Williamson et al., Proc.  
27 Natl. Acad. Sci. USA, 90: 4141-4145; Marks et al.,  
28 1991, J. Mol. Biol. 222: 581-597).  
29  
30 Several publications describe this method. For example,  
31 US Patent No 5,403,484 describes production of a

1 chimeric protein formed from the viral coat protein and  
2 the peptide of interest. In this method at least a  
3 functional portion of a viral coat protein is required  
4 to cause display of the chimeric protein or a processed  
5 form thereof on the outer surface of the virus. In  
6 addition, US Patent No 5,571,698 describes a method for  
7 obtaining a nucleic acid encoding a binding protein, a  
8 key component of which comprises preparing a population  
9 of amplifiable genetic packages which have a  
10 genetically determined outer surface protein, to cause  
11 the display of the potential binding domain on the  
12 outer surface of the genetic package. The genetic  
13 packages are selected from the group consisting of  
14 cells, spores and viruses. For example when the  
15 genetic package is a bacterial cell, the outer surface  
16 transport signal is derived from a bacterial outer  
17 surface protein, and when the genetic package is a  
18 filamentous bacteriophage, the outer surface transport  
19 signal is provided by the gene pIII (minor coat  
20 protein) or pVIII (major coat protein) of the  
21 filamentous phage.

22  
23 WO-A-92/01047 and WO-A-92/20791 describe methods for  
24 producing multimeric specific binding pairs, by  
25 expressing a first polypeptide chain fused to a viral  
26 coat protein, such as the gene pIII protein, of a  
27 secreted replicable genetic display package (RGDP)  
28 which displays a polypeptide at the surface of the  
29 package, and expressing a second polypeptide chain of  
30 the multimer, and allowing the two chains to come  
31 together as part of the RGDP.

1  
2     LacI fusion plasmid display. This method is based on  
3     the DNA binding ability of the lac repressor. Libraries  
4     of random peptides are fused to the lacI repressor  
5     protein, normally to the C-terminal end, through  
6     expression from a plasmid vector carrying the fusion  
7     gene. Linkage of the LacI-peptide fusion to its  
8     encoding DNA occurs via the lacO sequences on the  
9     plasmid, forming a stable peptide-LacI-peptide complex.  
10    These complexes are released from their host bacteria  
11    by cell lysis, and peptides of interest isolated by  
12    affinity purification on an immobilised target. The  
13    plasmids thus isolated can then be reintroduced into *E.*  
14    *coli* by electroporation to amplify the selected  
15    population for additional rounds of screening (Cull, M.  
16    G. et al. 1992. Proc. Natl. Acad. Sci. U.S.A. 89:1865-  
17    1869).

18  
19    US Patent No 5498530 describes a method for  
20    constructing a library of random peptides fused to a  
21    DNA binding protein in appropriate host cells and  
22    culturing the host cells under conditions suitable for  
23    expression of the fusion proteins intra-cellularly, in  
24    the cytoplasm of the host cells. This method also  
25    teaches that the random peptide is located at the  
26    carboxy terminus of the fusion protein and that the  
27    fusion protein-DNA complex is released from the host  
28    cell by cell lysis. No method is described for the  
29    protection of the DNA from degradation once released  
30    from the lysed cell. Several DNA binding proteins are  
31    claimed but no examples are shown except lacI.

1  
2 There remains a need for methods of constructing  
3 peptide libraries in addition to the methods described  
4 above. For instance, the above methods do not permit  
5 production of secreted peptides with a free carboxy  
6 terminus. The present invention describes an  
7 alternative method for isolating peptides of interest  
8 from libraries and has significant advantages over the  
9 prior art methods.

10  
11 In general terms, the present invention provides a  
12 method for screening a nucleotide library (usually a  
13 DNA library) for a nucleotide sequence which encodes a  
14 target peptide of interest. The method involves  
15 physically linking each peptide to a polynucleotide  
16 including the specific nucleotide sequence encoding  
17 that peptide. Linkage of a peptide to its encoding  
18 nucleotide sequence is achieved via linkage of the  
19 peptide to a nucleotide binding domain. A bifunctional  
20 chimeric protein with a nucleotide binding domain and a  
21 library member or target peptide (preferably with a  
22 function of interest) is thus obtained. The peptide of  
23 interest is bound to the polynucleotide encoding that  
24 peptide via the nucleotide binding domain of the  
25 chimeric protein.

26  
27 The polynucleotide-chimeric protein complex is then  
28 incorporated within a peptide display carrier package  
29 (PDCP), protecting the polynucleotide from subsequent  
30 degradation, while displaying the target peptide

1 portion on the outer surface of the peptide display  
2 carrier package (PDCP).

3

4 Thus, in one aspect, the present invention provides a  
5 peptide display carrier package (PDCP), said package  
6 comprising a polynucleotide-chimeric protein complex  
7 wherein the chimeric protein has a nucleotide binding  
8 portion and a target peptide portion, wherein said  
9 polynucleotide comprises a nucleotide sequence motif  
10 which is specifically bound by said nucleotide binding  
11 portion, and wherein at least the chimeric protein  
12 encoding portion of the polynucleotide not bound by the  
13 nucleotide binding portion of the chimeric protein is  
14 protected.

15

16 In one embodiment the polynucleotide is protected by a  
17 protein which binds non-specifically to naked  
18 polynucleotide. Examples include viral coat proteins,  
19 many of which are well-known in the art. Where the  
20 chosen viral coat protein requires an initiation  
21 sequence to commence general binding to the  
22 polynucleotide, this will be provided on the  
23 polynucleotide at appropriate location(s). A preferred  
24 coat protein is coat protein from a bacteriophage,  
25 especially M13.

26

27 Generally, the nucleic binding portion of the chimeric  
28 protein is selected for its specificity for the  
29 nucleotide sequence motif present in the recombinant  
30 polynucleotide encoding the chimeric protein.

31

1 Optionally, the nucleotide sequence motif may be an  
2 integral part of the protein encoding region of the  
3 polynucleotide. Alternatively, and more usually, the  
4 motif may be present in a non-coding region of the  
5 polynucleotide. For the purposes of this invention,  
6 all that is required is for the motif to be located on  
7 the polynucleotide such that the nucleotide binding  
8 portion of the chimeric protein is able to recognise  
9 and bind to it. Desirably the polynucleotide-chimeric  
10 protein complex has a dissociation constant of at least  
11 one hour.

12  
13 Optionally, the recombinant polynucleotide may comprise  
14 two or more nucleotide sequence motifs, each of which  
15 will be bound by a chimeric protein molecule.  
16 Preferably, the motifs are positioned along the length  
17 of the polynucleotide to avoid steric hindrance between  
18 the bound chimeric proteins.

19  
20 Preferably, the nucleotide sequence motif is not  
21 affected by the presence of additional nucleotide  
22 sequence (e.g. encoding sequence) at its 5' and/or 3'  
23 ends. Thus the chimeric fusion protein may include a  
24 target peptide portion at its N terminal end, at its C  
25 terminal end or may include two target peptide portions  
26 (which may be the same or different) at each end of the  
27 nucleotide binding portion, ie at both the N and C  
28 terminal ends of the chimeric protein. For example one  
29 target peptide may be an antibody of known specificity  
30 and the other peptide may be a peptide of potential  
31 interest.

1  
2 Desirably the target peptide portion of the chimeric  
3 protein is displayed externally on the peptide display  
4 carrier package, and is thus available for detection,  
5 reaction and/or binding.

6  
7 In more detail the PDCP may be composed two distinct  
8 elements:

- 9       a. A polynucleotide-chimeric protein complex. This  
10      links the displayed target peptide portion to the  
11      polynucleotide encoding that peptide portion  
12      through a specific polynucleotide binding portion.  
13      The nucleotide sequence encoding the chimeric  
14      protein, and the specific nucleotide sequence  
15      motif recognised by the nucleotide binding portion  
16      of the chimeric protein must be present on a  
17      segment of polynucleotide which can be  
18      incorporated into the PDCP; and  
19       b. A protective coat. This may be supplied by a  
20      replicable carrier or helper package capable of  
21      independent existence. Alternatively, a coat  
22      protein could be encoded by the recombinant  
23      polynucleotide of the invention. The protective  
24      coat for the polynucleotide-chimeric protein  
25      complex may be composed of a biological material  
26      such as protein or lipid, but the protective coat  
27      is not required for linking the target peptide to  
28      the polynucleotide encoding that peptide. The  
29      protective coat must allow the display of the  
30      target peptide portion of the chimeric protein on  
31      its outer surface. The carrier or helper package

1 may also provide the mechanism for releasing the  
2 intact PDCP from host cells when so required. By  
3 way of example, when a bacteriophage is the  
4 replicable carrier package, a protein coat of the  
5 bacteriophage surrounds the polynucleotide-  
6 chimeric protein complex to form the PDCP, which  
7 is then extruded from the host bacterial cell.

8

9 The invention described herein demonstrates that  
10 peptides fused to a nucleotide binding domain can be  
11 displayed externally, even through a bacteriophage  
12 carrier package protein coat, while still bound to the  
13 polynucleotide encoding the displayed peptide.

14

15 The present invention also provides a recombinant  
16 polynucleotide comprising a nucleotide sequence  
17 encoding a chimeric protein having a nucleotide binding  
18 portion operably linked to a target peptide portion,  
19 wherein said polynucleotide includes a specific  
20 nucleotide sequence motif which is bound by the  
21 nucleotide binding portion of said chimeric protein and  
22 further encoding a non-sequence-specific nucleotide  
23 binding protein.

24

25 Desirably, the recombinant polynucleotide is a  
26 recombinant expression system, able to express the  
27 chimeric protein when placed in a suitable environment,  
28 for example a compatible host cell. After its  
29 expression, the chimeric protein binds to the specific  
30 nucleotide sequence (motif) present in the

1 polynucleotide comprising the nucleotide sequence  
2 encoding the chimeric protein.

3

4 Optionally there may be a linker sequence located  
5 between the nucleotide sequence encoding the nucleotide  
6 binding portion and the polynucleotide inserted into  
7 the restriction enzyme site of the construct.

8

9 Desirably the nucleotide binding portion is a DNA  
10 binding domain of an estrogen or progesterone receptor,  
11 or a functional equivalent thereof. Examples of  
12 sequences encoding such nucleotide binding portions are  
13 set out in SEQ ID Nos 11 and 13.

14

15 The term "expression system" is used herein to refer to  
16 a genetic sequence which includes a protein-encoding  
17 region and is operably linked to all of the genetic  
18 signals necessary to achieve expression of that region.

19 Optionally, the expression system may also include  
20 regulatory elements, such as a promoter or enhancer to  
21 increase transcription and/or translation of the  
22 protein encoding region or to provide control over  
23 expression. The regulatory elements may be located  
24 upstream or downstream of the protein encoding region  
25 or within the protein encoding region itself. Where  
26 two or more distinct protein encoding regions are  
27 present these may use common regulatory element(s) or  
28 have separate regulatory element(s).

29

30 Generally, the recombinant polynucleotide described  
31 above will be DNA. Where the expression system is

1 based upon an M13 vector, usually the polynucleotide  
2 binding portion of the expressed chimeric portion will  
3 be single-stranded DNA. However, other vector systems  
4 may be used and the nucleotide binding portion may be  
5 selected to bind preferentially to double-stranded DNA  
6 or to double or single-stranded RNA, as convenient.

7

8 Additionally the present invention provides a vector  
9 containing such a recombinant expression system and  
10 host cells transformed with such a recombinant  
11 expression system (optionally in the form of a vector).

12

13 Whilst the recombinant polynucleotide described above  
14 forms an important part of the present invention, we  
15 are also concerned with the ability to screen large  
16 (e.g. of at least  $10^5$  members, for example  $10^6$  or even  
17  $10^7$  members) libraries of genetic material. One of the  
18 prime considerations therefore is the provision of a  
19 recombinant genetic construct into which each member of  
20 said library can individually be incorporated to form  
21 the recombinant polynucleotide described above and to  
22 express the chimeric protein thereby encoded (the  
23 target peptide of which is encoded by the nucleotide  
24 library member incorporated into the construct).

25

26 Thus viewed in a further aspect the present invention  
27 provides a genetic construct or set of genetic  
28 constructs comprising a polynucleotide having a  
29 sequence which includes:

30

- 1    i)    a sequence encoding a nucleotide binding portion  
2       able to recognise and bind to a specific sequence  
3       motif;
- 4    ii)   the sequence motif recognised and bound by the  
5       nucleotide binding portion encoded by (i);
- 6    iii) a restriction enzyme site which permits insertion  
7       of a polynucleotide, said site being designed to  
8       operably link said polynucleotide to the sequence  
9       encoding the nucleotide binding portion so that  
10      expression of the operably linked polynucleotide  
11      sequences yields a chimeric protein; and
- 12    iv) a sequence encoding a nucleotide binding protein  
13      which binds non-specifically to naked  
14      polynucleotide.

15  
16   Optionally there may be a linker sequence located  
17   between the nucleotide sequence encoding the nucleotide  
18   binding portion and the sequence of the polynucleotide  
19   from the library inserted into the restriction enzyme  
20   site of the construct.

21  
22   Desirably the nucleotide binding portion is a DNA  
23   binding domain of an estrogen or progesterone receptor,  
24   or a functional equivalent thereof. Examples of  
25   sequences encoding such nucleotide binding portions are  
26   set out in SEQ ID Nos 11 and 13.

27  
28   Suitable genetic constructs according to the invention  
29   include pDM12, pDM14 and pDM16, deposited at NCIMB on  
30   28 August 1998 under Nos NCIMB 40970, NCIMB 40971 and  
31   NCIMB 40972 respectively.

1  
2 It is envisaged that a conventionally produced genetic  
3 library may be exposed to the genetic construct(s)  
4 described above. Thus, each individual member of the  
5 genetic library will be separately incorporated into  
6 the genetic construct and the library will be present  
7 in the form of a library of recombinant polynucleotides  
8 (as described above), usually in the form of vectors,  
9 each recombinant polynucleotide including as library  
10 member.

11  
12 Thus, in a further aspect, the present invention  
13 provides a library of recombinant polynucleotides (as  
14 defined above) wherein each polynucleotide includes a  
15 polynucleotide obtained from a genetic library and  
16 which encodes the target peptide portion of the  
17 chimeric protein expressed by the recombinant  
18 polynucleotide.

19  
20 Optionally, the chimeric protein may further include a  
21 linker sequence located between the nucleotide binding  
22 portion and the target peptide portion. The linker  
23 sequence will reduce steric interference between the  
24 two portions of the protein. Desirably the linker  
25 sequence exhibits a degree of flexibility.

26  
27 Also disclosed are methods for constructing and  
28 screening libraries of PDCP particles, displaying many  
29 different peptides, allowing the isolation and  
30 identification of particular peptides by means of  
31 affinity techniques relying on the binding activity of

1 the peptide of interest. The resulting polynucleotide  
2 sequences can therefore be more readily identified, re-  
3 cloned and expressed.

4

5 A method of constructing a genetic library, said method  
6 comprising:

7

8 a) constructing multiple copies of a recombinant  
9 vector comprising a polynucleotide sequence which  
10 encodes a nucleotide binding portion able to  
11 recognise and bind to a specific sequence motif  
12 (and optionally also including the specific  
13 sequence motif);

14

15 b) operably linking each said vector to a  
16 polynucleotide encoding a target polypeptide, such  
17 that expression of said operably linked vector  
18 results in expression of a chimeric protein  
19 comprising said target peptide and said nucleotide  
20 binding portions; wherein said multiple copies of  
21 said operably linked vectors collectively express  
22 a library of target peptide portions;

23

24 c) transforming host cells with the vectors of step  
25 b);

26

27 d) culturing the host cells of step c) under  
28 conditions suitable for expression of said  
29 chimeric protein;

30

1   e)   providing a recombinant polynucleotide comprising  
2   the nucleotide sequence motif specifically  
3   recognised by the nucleotide binding portion and  
4   exposing this polynucleotide to the chimeric  
5   protein of step d) to yield a polynucleotide-  
6   chimeric protein complex; and

7

8   f)   causing production of a non-sequence-specific  
9   moiety able to bind to the non-protected portion  
10   of the polynucleotide encoding the chimeric  
11   protein to form a peptide display carrier package.

12

13   The present invention further provides a method of  
14   screening a genetic library, said method comprising:

15

16   a)   exposing the polynucleotide members of said  
17   library to multiple copies of a genetic construct  
18   comprising a nucleotide sequence encoding a  
19   nucleotide binding portion able to recognise and  
20   bind to a specific sequence motif, under  
21   conditions suitable for the polynucleotides of  
22   said library each to be individually ligated into  
23   one copy of said genetic construct, to create a  
24   library of recombinant polynucleotides;

25

26   b)   exposing said recombinant polynucleotides to a  
27   population of host cells, under conditions  
28   suitable for transformation of said host cells by  
29   said recombinant polynucleotides;

30

31   c)   selecting for transformed host cells;

- 1
- 2     d) exposing said transformed host cells to conditions
- 3       suitable for expression of said recombinant
- 4       polynucleotide to yield a chimeric protein; and
- 5
- 6     e) providing a recombinant polynucleotide comprising
- 7       the nucleotide sequence motif specifically
- 8       recognised by the nucleotide binding portion and
- 9       exposing this polynucleotide to the chimeric
- 10      protein of step d) to yield a polynucleotide-
- 11      chimeric protein complex;
- 12
- 13    f) protecting any exposed portions of the
- 14      polynucleotide in the complex of step e) to form a
- 15      peptide display carrier package; and
- 16
- 17    g) screening said peptide display carrier package to
- 18      select only those packages displaying a target
- 19      peptide portion having the characteristics
- 20      required.
- 21
- 22    Desirably in step a) the genetic construct is pDM12,
- 23    pDM14 or pDM16.
- 24
- 25    Desirably in step f) the peptide display package
- 26    carrier is extruded from the transformed host cell
- 27    without lysis of the host cell.
- 28
- 29    Generally the transformed host cells will be plated out
- 30    or otherwise divided into single colonies following

1 transformation and prior to expression of the chimeric  
2 protein.

3

4 The screening step g) described above may look for a  
5 particular target peptide either on the basis of  
6 function (e.g. enzymic activity) or structure (e.g.  
7 binding to a specific antibody). Once the peptide  
8 display carrier package is observed to include a target  
9 peptide with the desired characteristics, the  
10 polynucleotide portion thereof (which of course encodes  
11 the chimeric protein itself) can be amplified, cloned  
12 and otherwise manipulated using standard genetic  
13 engineering techniques.

14

15 The current invention differs from the prior art  
16 teaching of the previous disclosures US Patent No  
17 5,403,484 and US Patent No 5,571,698, as the invention  
18 does not require outer surface transport signals, or  
19 functional portions of viral coat proteins, to enable  
20 the display of chimeric binding proteins on the outer  
21 surface of the viral particle or genetic package.

22

23 The current invention also differs from the teaching of  
24 WO-A-92/01047 and WO-A-92/20791, as no component of a  
25 secreted replicable genetic display package, or viral  
26 coat protein is required, to enable display of the  
27 target peptide on the outer surface of the viral  
28 particle.

29

30 The current invention differs from the teaching of US  
31 Patent No 5498530, as it enables the display of

1 chimeric proteins, linked to the polynucleotide  
2 encoding the chimeric protein, extra-cellularly, not in  
3 the cytoplasm of a host cell. In the current invention  
4 the chimeric proteins are presented on the outer  
5 surface of a peptide display carrier package (PDCP)  
6 which protects the DNA encoding the chimeric protein,  
7 and does not require cell lysis to obtain access to the  
8 chimeric protein-DNA complex. Finally, the current  
9 invention does not rely upon the lacI DNA binding  
10 protein to form the chimeric protein-DNA complex.

11

12 In one embodiment of the invention, the nucleotide  
13 binding portion of the chimeric protein comprises a DNA  
14 binding domain from one or more of the nuclear steroid  
15 receptor family of proteins, or a functional equivalent  
16 of such a domain. Particular examples include (but are  
17 not limited to) a DNA binding domain of the estrogen  
18 receptor or the progesterone receptor, or functional  
19 equivalents thereof. These domains can recognise  
20 specific DNA sequences, termed hormone response  
21 elements (HRE), which can be bound as both double and  
22 single-stranded DNA. The DNA binding domain of such  
23 nuclear steroid receptor proteins is preferred.

24

25 The estrogen receptor is especially referred to below  
26 by way of example, for convenience since:

27 (a) The estrogen receptor is a large multifunctional  
28 polypeptide of 595 amino acids which functions in the  
29 cytoplasm and nucleus of eukaryotic cells (Green et  
30 al., 1986, Science 231: 1150-1154). A minimal high  
31 affinity DNA binding domain (DBD) has been defined

1 between amino acids 176 and 282 (Mader et al., 1993,  
2 Nucleic Acids Res. 21: 1125-1132). The functioning of  
3 this domain (i.e. DNA binding) is not inhibited by the  
4 presence of non-DNA binding domains at both the N and C  
5 terminal ends of this domain, in the full length  
6 protein.

7

8 (b) The estrogen receptor DNA binding domain fragment  
9 (amino acids 176-282) has been expressed in *E. coli* and  
10 shown to bind to the specific double stranded DNA  
11 estrogen receptor target HRE nucleotide sequence, as a  
12 dimer with a similar affinity (0.5nM) to the parent  
13 molecule (Murdoch et al. 1990, Biochemistry 29: 8377-  
14 8385; Mader et al., 1993, Nucleic Acids Research 21:  
15 1125-1132). DBD dimerization on the surface of the PDCP  
16 should result in two peptides displayed per particle.  
17 This bivalent display can aid in the isolation of low  
18 affinity peptides and peptides that are required to  
19 form a bivalent conformation in order to bind to a  
20 particular target, or activate a target receptor. The  
21 estrogen receptor is capable of binding to its 38 base  
22 pair target HRE sequence, consensus sequence:

23

- 24 1) 5'-TCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3'  
25 ("minus strand") SEQ ID No 77, and  
26 2) 3'-AGTCCAGTCTCACTGGACTCGATTATTGTGTAAGTC-5'  
27 ("plus strand") SEQ ID No 78,

28

29 with high affinity and specificity, under the salt and  
30 pH conditions normally required for selection of  
31 binding peptides. Moreover, binding affinity is

1 increased 60-fold for the single-stranded coding, or  
2 "plus", strand (i.e. SEQ ID No 78) of the HRE  
3 nucleotide sequence over the double stranded form of  
4 the specific target nucleotide sequence (Peale et al.  
5 1988, Proc. Natl. Acad. Sci. USA 85: 1038-1042;  
6 Lannigan & Notides, 1989, Proc. Natl. Acad. Sci. USA  
7 86: 863-867).

8

9 In an embodiment of the invention where the DNA binding  
10 component of the peptide display carrier package is the  
11 estrogen receptor, the nucleotide (DNA) binding portion  
12 contains a minimum sequence of amino acids 176-282 of  
13 the estrogen receptor protein. In addition, the  
14 consensus estrogen receptor target HRE sequence is  
15 cloned in such a way that if single stranded DNA can be  
16 produced then the coding, or "plus", strand of the  
17 estrogen receptor HRE nucleotide sequence is  
18 incorporated into single-stranded DNA. An example of a  
19 vector suitable for this purpose is pUC119 (see Viera  
20 et al., Methods in Enzymology, Vol 153, pages 3-11,  
21 1987).

22

23 In a preferred embodiment of the invention a peptide  
24 display carrier package (PDCP) can be assembled when a  
25 bacterial host cell is transformed with a bacteriophage  
26 vector, which vector comprises a recombinant  
27 polynucleotide as described above. The expression  
28 vector will also comprise the specific nucleotide motif  
29 that can be bound by the nucleotide binding portion of  
30 the chimeric protein. Expression of recombinant  
31 polynucleotide results in the production of the

1 chimeric protein which comprises the target peptide and  
2 the nucleotide binding portion. The host cell is grown  
3 under conditions suitable for chimeric protein  
4 expression and assembly of the bacteriophage particles,  
5 and the association of the chimeric protein with the  
6 specific nucleotide sequence in the expression vector.  
7 In this embodiment, since the vector is a  
8 bacteriophage, which replicates to produce a single-  
9 stranded DNA, the nucleotide binding portion preferably  
10 has an affinity for single-stranded DNA. Incorporation  
11 of the vector single-stranded DNA-chimeric protein  
12 complex into bacteriophage particles results in the  
13 assembly of the peptide display carrier package (PDCP),  
14 and display of the target peptide on the outer surface  
15 of the PDCP.

16

17 In this embodiment both of the required elements for  
18 producing peptide display carrier packages are  
19 contained on the same vector. Incorporation of the DNA-  
20 chimeric protein complex into a peptide display carrier  
21 package (PDCP) is preferred as DNA degradation is  
22 prevented, large numbers of PDCPs are produced per host  
23 cell, and the PDCPs are easily separated from the host  
24 cell without recourse to cell lysis.

25

26 In a more preferred embodiment, the vector of the is a  
27 phagemid vector (for example pUC119) where expression  
28 of the chimeric protein is controlled by an inducible  
29 promoter. In this embodiment the PDCP can only be  
30 assembled following infection of the host cell with  
31 both phagemid vector and helper phage. The transfected

1 host cell is then cultivated under conditions suitable  
2 for chimeric protein expression and assembly of the  
3 bacteriophage particles.

4

5 In this embodiment the elements of the PDCP are  
6 provided by two separate vectors. The phagemid derived  
7 PDCP is superior to phagemid derived display packages  
8 disclosed in WO-A-92/01047 where a proportion of  
9 packages displaying bacteriophage coat protein fusion  
10 proteins will contain the helper phage DNA, not the  
11 fusion protein DNA sequence. In the current invention,  
12 a PDCP can display the chimeric fusion protein only  
13 when the package contains the specific nucleotide motif  
14 recognised by the nucleotide binding portion. In most  
15 embodiments this sequence will be present on the same  
16 DNA segment that encodes the fusion protein. In  
17 addition, the prior art acknowledges that when mutant  
18 and wild type proteins are co-expressed in the same  
19 bacterial cell, the wild type protein is produced  
20 preferentially. Thus, when the wild type helper phage,  
21 phage display system of WO-A-92/01047 is used, both  
22 wild type gene pIII and target peptide-gene pIII  
23 chimeric proteins are produced in the same cell. The  
24 result of this is that the wild type gene pIII protein  
25 is preferentially packaged into bacteriophage  
26 particles, over the chimeric protein. In the current  
27 invention, there is no competition with wild type  
28 bacteriophage coat proteins for packaging.

29

30 Desirably the target peptide is displayed in a location  
31 exposed to the external environment of the PDCP, after

1 the PDCP particle has been released from the host cell  
2 without recourse to cell lysis. The target peptide is  
3 then accessible for binding to its ligand. Thus, the  
4 target peptide may be located at or near the N-terminus  
5 or the C-terminus of a nucleotide binding domain, for  
6 example the DNA binding domain of the estrogen  
7 receptor.

8

9 The present invention also provides a method for  
10 screening a DNA library expressing one or more  
11 polypeptide chains that are processed, folded and  
12 assembled in the periplasmic space to achieve  
13 biological activity. The PDCP may be assembled by the  
14 following steps:

15

16 (a) Construction of N- or C-terminal DBD chimeric  
17 protein fusions in a phagemid vector.

18 (i) When the target peptide is located at the N-  
19 terminus of the nucleotide binding portion, a library  
20 of DNA sequences each encoding a potential target  
21 peptide is cloned into an appropriate location of an  
22 expression vector (i.e. behind an appropriate promoter  
23 and translation sequences and a sequence encoding a  
24 signal peptide leader directing transport of the  
25 downstream fusion protein to the periplasmic space) and  
26 upstream of the sequence encoding the nucleotide  
27 binding portion. In a preferred embodiment the DNA  
28 sequence(s) of interest may be joined, by a region of  
29 DNA encoding a flexible amino acid linker, to the 5'-  
30 end of an estrogen receptor DBD.

1               (ii) Alternatively, when the target peptide is  
2 located at the C-terminus of the nucleotide binding  
3 domain, a library of DNA sequences each encoding a  
4 potential target peptide is cloned into the expression  
5 vector so that the nucleotide sequence coding for the  
6 nucleotide binding portion is upstream of the cloned  
7 DNA target peptide encoding sequences, said nucleotide  
8 binding portion being positioned behind an appropriate  
9 promoter and translation sequences and a sequence  
10 encoding a signal peptide leader directing transport of  
11 the downstream fusion protein to the periplasmic space.  
12 In a preferred embodiment, DNA sequence(s) of interest  
13 may be joined, by a region of DNA encoding a flexible  
14 amino acid linker estrogen receptor DBD DNA sequence.

15  
16 Located on the expression vector is the specific HRE  
17 nucleotide sequence recognised, and bound, by the  
18 estrogen receptor DBD. In order to vary the number of  
19 chimeric proteins displayed on each PDCP particle, this  
20 sequence can be present as one or more copies in the  
21 vector.

22  
23 (b) Incorporation into the PDCP. Non-lytic helper  
24 bacteriophage infects host cells containing the  
25 expression vector. Preferred types of bacteriophage  
26 include the filamentous phage fd, f1 and M13. In a  
27 more preferred embodiment the bacteriophage may be  
28 M13K07.

29  
30 The protein(s) of interest are expressed and  
31 transported to the periplasmic space, and the properly

1 assembled proteins are incorporated into the PDCP  
2 particle by virtue of the high affinity interaction of  
3 the DBD with the specific target nucleotide sequence  
4 present on the phagemid vector DNA which is naturally  
5 packaged into phage particles in a single-stranded  
6 form. The high affinity interaction between the DBD  
7 protein and its specific target nucleotide sequence  
8 prevents displacement by bacteriophage coat proteins  
9 resulting in the incorporation of the protein(s) of  
10 interest onto the surface of the PDCP as it is extruded  
11 from the cell.

12

13 (c) Selection of the peptide of interest. Particles  
14 which display the peptide of interest are then selected  
15 from the culture by affinity enrichment techniques.  
16 This is accomplished by means of a ligand specific for  
17 the protein of interest, such as an antigen if the  
18 protein of interest is an antibody. The ligand may be  
19 presented on a solid surface such as the surface of an  
20 ELISA plate, or in solution. Repeating the affinity  
21 selection procedure provides an enrichment of clones  
22 encoding the desired sequences, which may then be  
23 isolated for sequencing, further cloning and/or  
24 expression.

25

26 Numerous types of libraries of peptides fused to the  
27 DBD can be screened under this embodiment including:

28

29 (i) Random peptide sequences encoded by synthetic  
30 DNA of variable length.

31

1       (ii) Single-chain Fv antibody fragments. These  
2       consist of the antibody heavy and light chain  
3       variable region domains joined by a flexible  
4       linker peptide to create a single-chain antigen  
5       binding molecule.

6

7       (iii) Random fragments of naturally occurring  
8       proteins isolated from a cell population  
9       containing an activity of interest.

10

11      In another embodiment the invention concerns methods  
12     for screening a DNA library whose members require more  
13     than one chain for activity, as required by, for  
14     example, antibody Fab fragments for ligand binding. In  
15     this embodiment heavy or light chain antibody DNA is  
16     joined to a nucleotide sequence encoding a DNA binding  
17     domain of, for example, the estrogen receptor in a  
18     phagemid vector. Typically the antibody DNA library  
19     sequences for either the heavy (VH and CH1) or light  
20     chain (VL and CL) genes are inserted in the 5' region  
21     of the estrogen receptor DBD DNA, behind an appropriate  
22     promoter and translation sequences and a sequence  
23     encoding a signal peptide leader directing transport of  
24     the downstream fusion protein to the periplasmic space.

25

26      Thus, a DBD fused to a DNA library member-encoded  
27     protein is produced and assembled in to the viral  
28     particle after infection with bacteriophage. The second  
29     and any subsequent chain(s) are expressed separately  
30     either:

31

1       (a) from the same phagemid vector containing the DBD  
2 and the first polypeptide fusion protein,  
3 or

4  
5       (b) from a separate region of DNA which may be present  
6 in the host cell nucleus, or on a plasmid, phagemid or  
7 bacteriophage expression vector that can co-exist, in  
8 the same host cell, with the first expression vector,  
9 so as to be transported to the periplasm where they  
10 assemble with the first chain that is fused to the DBD  
11 protein as it exits the cell. Peptide display carrier  
12 packages (PDCP) which encode the protein of interest  
13 can then be selected by means of a ligand specific for  
14 the protein.

15  
16 In yet another embodiment, the invention concerns  
17 screening libraries of bi-functional peptide display  
18 carrier packages where two or more activities of  
19 interest are displayed on each PDCP. In this  
20 embodiment, a first DNA library sequence(s) is inserted  
21 next to a first DNA binding domain (DBD) DNA sequence,  
22 for example the estrogen receptor DBD, in an  
23 appropriate vector, behind an appropriate promoter and  
24 translation sequences and a sequence encoding a signal  
25 peptide leader directing transport of this first  
26 chimeric protein to the periplasmic space. A second  
27 chimeric protein is also produced from the same, or  
28 separate, vector by inserting a second DNA library  
29 sequence(s) next to a second DBD DNA sequence which is  
30 different from the first DBD DNA sequence, for example  
31 the progesterone receptor DBD, behind an appropriate

1 promoter and translation sequences and a sequence  
2 encoding a signal peptide leader. The first, or only,  
3 vector contains the specific HRE nucleotide sequences  
4 for both estrogen and progesterone receptors.  
5 Expression of the two chimeric proteins, results in a  
6 PDCP with two different chimeric proteins displayed. As  
7 an example, one chimeric protein could possess a  
8 binding activity for a particular ligand of interest,  
9 while the second chimeric protein could possess an  
10 enzymatic activity. Binding by the PDCP to the ligand  
11 of the first chimeric protein could then be detected by  
12 subsequent incubation with an appropriate substrate for  
13 the second chimeric protein. In an alternative  
14 embodiment a bi-functional PDCP may be created using a  
15 single DBD, by cloning one peptide at the 5'-end of the  
16 DBD, and a second peptide at the 3'-end of the DBD.  
17 Expression of this single bi-functional chimeric  
18 protein results in a PDCP with two different  
19 activities.

20

21 We have investigated the possibility of screening  
22 libraries of peptides, fused to a DNA binding domain  
23 and displayed on the surface of a display package, for  
24 particular peptides with a biological activity of  
25 interest and recovering the DNA encoding that activity.  
26 Surprisingly, by manipulating the estrogen receptor DNA  
27 binding domain in conjunction with M13 bacteriophage we  
28 have been able to construct novel particles which  
29 display large biologically functional molecules, that  
30 allows enrichment of particles with the desired  
31 specificity.

1  
2     The invention described herein provides a significant  
3     breakthrough in DNA library screening technology.

4  
5     The invention will now be further described by  
6     reference to the non-limiting examples and figures  
7     below.

8

9     **Description of Figures**

10  
11    Figure 1 shows the pDM12 N-terminal fusion estrogen  
12    receptor DNA binding domain expression vector  
13    nucleotide sequence (SEQ ID No 1), between the HindIII  
14    and EcoRI restriction sites, comprising a *pelB* leader  
15    secretion sequence (in italics) (SEQ ID No 2), multiple  
16    cloning site containing *SfiI* and *NotI* sites, flexible  
17    (glycine)<sub>4</sub>-serine linker sequence (boxed), a fragment of  
18    the estrogen receptor gene comprising amino acids 176-  
19    282 (SEQ ID No 3) of the full length molecule, and the  
20    38 base pair consensus estrogen receptor DNA binding  
21    domain HRE sequence.

22

23    Figure 2 shows the OD<sub>450nm</sub> ELISA data for negative  
24    control M13K07 phage, and single-clone PDCP display  
25    culture supernatants (#1-4, see Example 3) isolated by  
26    selection of the lymphocyte cDNA-pDM12 library against  
27    anti-human immunoglobulin kappa antibody.

28

29    Figure 3 shows partial DNA (SEQ ID No 4) and amino acid  
30    (SEQ ID No 5) sequence for the human immunoglobulin  
31    kappa constant region (Kabat, E. A. et al., Sequences

1 of Proteins of Immunological Interest. 4<sup>th</sup> edition. U.S.  
2 Department of Health and Human Services. 1987), and  
3 ELISA positive clones #2 (SEQ ID Nos 6 and 7) and #3  
4 (SEQ ID Nos 8 and 9) from Figure 2 which confirms the  
5 presence of human kappa constant region DNA in-frame  
6 with the pelB leader sequence (pelB leader sequence is  
7 underlined, the leader sequence cleavage site is  
8 indicated by an arrow). The differences in the 5'-end  
9 sequence demonstrates that these two clones were  
10 selected independently from the library stock. The PCR  
11 primer sequence is indicated in bold, clone #2 was  
12 originally amplified with CDNAPCRBAK1 and clone #3 was  
13 amplified with CDNAPCRBAK2.

14

15 Figure 4 shows the pDM14 N-terminal fusion estrogen  
16 receptor DNA binding domain expression vector  
17 nucleotide sequence (SEQ ID No 10), between the HindIII  
18 and EcoRI restriction sites, comprising a pelB leader  
19 secretion sequence (in italics) (SEQ ID No 11), multiple  
20 cloning site containing SfiI and NotI sites, flexible  
21 (glycine)<sub>4</sub>-serine linker sequence (boxed), a fragment of  
22 the estrogen receptor gene comprising amino acids 176-  
23 282 (see SEQ ID No 12) of the full length molecule, and  
24 the two 38 base pair estrogen receptor DNA binding  
25 domain HRE sequences (HRE 1 and HRE 2).

26

27 Figure 5 shows the pDM16 C-terminal fusion estrogen  
28 receptor DNA binding domain expression vector  
29 nucleotide sequence (SEQ ID No 13), between the HindIII  
30 and EcoRI restriction sites, comprising a pelB leader  
secretion sequence (in italics), a fragment of the

1 estrogen receptor gene comprising amino acids 176-282  
2 (SEQ ID No 14) of the full length molecule, flexible  
3 (glycine)<sub>4</sub>-serine linker sequence (boxed), multiple  
4 cloning site containing SfiI and NotI sites and the 38  
5 base pair estrogen receptor DNA binding domain HRE  
6 sequence.

7

8 Figure 6 shows the OD<sub>450nm</sub> ELISA data for N-cadherin-  
9 pDM16 C-terminal display PDCP binding to anti-pan-  
10 cadherin monoclonal antibody in serial dilution ELISA  
11 as ampicillin resistance units (a.r.u.). Background  
12 binding of negative control M13K07 helper phage is also  
13 shown.

14

15 Figure 7 shows the OD<sub>450nm</sub> ELISA data for *in vivo*  
16 biotinylated PCC-pDM16 C-terminal display PDCP binding  
17 to streptavidin in serial dilution ELISA as ampicillin  
18 resistance units (a.r.u.). Background binding of  
19 negative control M13K07 helper phage is also shown.

20

21 Figure 8 shows the OD<sub>450nm</sub> ELISA data for a human scFv  
22 PDCP isolated from a human scFv PDCP display library  
23 selected against substance P. The PDCP was tested  
24 against streptavidin (1), streptavidin-biotinylated  
25 substance P (2), and streptavidin-biotinylated CGRP  
26 (3), in the presence (B) or absence (A) of free  
27 substance P.

28

29 Figure 9 shows the DNA (SEQ ID Nos 15 and 17) and amino  
30 acid (SEQ ID No 16 and 18) sequence of the substance P  
31 binding scFv isolated from a human scFv PDCP display

1 library selected against substance P. Heavy chain (SEQ  
2 ID Nos 15 and 16) and light chain (SEQ ID Nos 17 and  
3 18) variable region sequence is shown with the CDRs  
4 underlined and highlighted in bold.

5

6 **Materials and Methods**

7 The following procedures used by the present applicant  
8 are described in Sambrook, J., et al., 1989 supra.:  
9 restriction enzyme digestion, ligation, preparation of  
10 electrocompetent cells, electroporation, analysis of  
11 restriction enzyme digestion products on agarose gels,  
12 DNA purification using phenol/chloroform, preparation  
13 of 2xTY medium and plates, preparation of ampicillin,  
14 kanamycin, IPTG (Isopropyl  $\beta$ -D-Thiogalactopyranoside)  
15 stock solutions, and preparation of phosphate buffered  
16 saline.

17

18 Restriction enzymes, T4 DNA ligase and cDNA synthesis  
19 reagents (Superscript plasmid cDNA synthesis kit) were  
20 purchased from Life Technologies Ltd (Paisley,  
21 Scotland, U.K.). Oligonucleotides were obtained from  
22 Cruachem Ltd (Glasgow, Scotland, U.K.), or Genosys  
23 Biotechnologies Ltd (Cambridge, U.K.). Taq DNA  
24 polymerase, Wizard SV plasmid DNA isolation kits,  
25 streptavidin coated magnetic beads and mRNA isolation  
26 reagents (PolyATract 1000) were obtained from Promega  
27 Ltd (Southampton, Hampshire, U.K.). Taqplus DNA  
28 polymerase was obtained from Stratagene Ltd (Cambridge,  
29 U.K.). PBS, BSA, streptavidin, substance P and anti-pan  
30 cadherin antibody were obtained from SIGMA Ltd (Poole,  
31 Dorset, U.K.). Anti-M13-HRP conjugated antibody,

1 Kanamycin resistant M13K07 helper bacteriophage and  
2 RNAGuard were obtained from Pharmacia Ltd (St. Albans,  
3 Herts, U.K.) and anti-human Igκ antibody from Harlan-  
4 Seralab (Loughborough, Leicestershire, U.K.)  
5 Biotinylated substance P and biotinylated calcitonin  
6 gene related peptide (CGRP) were obtained from  
7 Peninsula Laboratories (St. Helens, Merseyside, U.K.).  
8  
9 Specific embodiments of the invention are given below  
10 in Examples 1 to 9.  
11

1   **Example 1. Construction of a N-terminal PDCP display**  
2   **phagemid vector pDM12.**

3  
4   The pDM12 vector was constructed by inserting an  
5   estrogen receptor DNA binding domain, modified by  
6   appropriate PCR primers, into a phagemid vector pDM6.  
7   The pDM6 vector is based on the pUC119 derived phage  
8   display vector pHEN1 (Hoogenboom et al., 1991, Nucleic  
9   Acids Res. 19: 4133-4137). It contains (Gly)<sub>4</sub>Ser linker,  
10   Factor Xa cleavage site, a full length gene III, and  
11   streptavidin tag peptide sequence (Schmidt, T.G. and  
12   Skerra, A., 1993, Protein Engineering 6: 109-122), all  
13   of which can be removed by NotI-EcoRI digestion and  
14   agarose gel electrophoresis, leaving a pelB leader  
15   sequence, SfiI, NcoI and PstI restriction sites  
16   upstream of the digested NotI site. The cloned DNA  
17   binding domain is under the control of the lac promoter  
18   found in pUC119.

19  
20   **Preparation of pDM6**

21  
22   The pDM12 vector was constructed by inserting an  
23   estrogen receptor DNA binding domain, modified by  
24   appropriate PCR primers, into a phagemid vector pDM6.  
25   The pDM6 vector is based on the gene pIII phage display  
26   vector pHEN1 (Hoogenboom et al., 1991, Nucleic Acids  
27   Res. 19: 4133-4137), itself derived from pUC119 (Viera,  
28   J. and Messing, J., 1987, Methods in Enzymol. 153:  
29   3-11). It was constructed by amplifying the pIII gene  
30   in pHEN1 with two oligonucleotides:

31

1 PDM6BAK: 5 -TTT TCT GCA GTA ATA GGC GGC CGC AGG GGG AGG  
2 AGG GTC CAT CGA AGG TCG CGA AGC AGA GAC TGT TGA AAG T-3  
3 (SEQ ID No 19) and

4

5 PDM6FOR: 5 - TTT TGA ATT CTT ATT AAC CAC CGA ACT GCG  
6 GGT GAC GCC AAG CGC TTG CGG CCG TTA AGA CTC CTT ATT ACG  
7 CAG-3 (SEQ ID No 20).

8

9 and cloning the PstI-EcoRI digested PCR product back  
10 into similarly digested pHEN1, thereby removing the  
11 c-myc tag sequence and supE TAG codon from pHEN1. The  
12 pDM6 vector contains a (Gly)<sub>4</sub>Ser linker, Factor Xa  
13 cleavage site, a full length gene III, and streptavidin  
14 tag peptide sequence (Schmidt, T.G. and Skerra, A.,  
15 1993, Protein Engineering 6: 109-122), all of which can  
16 be removed by NotI-EcoRI digestion and agarose gel  
17 electrophoresis, leaving a pelB leader sequence, SfiI,  
18 NcoI and PstI restriction sites upstream of the  
19 digested NotI site. The cloned DNA binding domain is  
20 under the control of the lac promoter found in pUC119.

21

22 The estrogen receptor DNA binding domain was isolated  
23 from cDNA prepared from human bone marrow (Clontech,  
24 Palo Alto, California, U.S.A.). cDNA can be prepared by  
25 many procedures well known to those skilled in the art.  
26 As an example, the following method using a Superscript  
27 plasmid cDNA synthesis kit can be used:

28

29 **(a) First strand synthesis.**

30

1    5 $\mu$ g of bone marrow mRNA, in 5 $\mu$ l DEPC-treated water was  
2    thawed on ice and 2 $\mu$ l (50pmol) of cDNA synthesis primer  
3    (5'-AAAAGCGGCCGCACTGGCCTGAGAGA(N)<sub>6</sub>-3') (SEQ ID No 21)  
4    was added to the mRNA and the mixture heated to 70°C for  
5    10 minutes, then snap-chilled on ice and spun briefly  
6    to collect the contents to the bottom of the tube. The  
7    following were then added to the tube:

8	1000u/ml RNAGuard	1 $\mu$ l
9	5x first strand buffer	4 $\mu$ l
10	0.1M DTT	2 $\mu$ l
11	10mM dNTPs	1 $\mu$ l
12	200u/ $\mu$ l SuperScript II reverse transcriptase 5 $\mu$ l	
13	The mixture was mixed by pipetting gently and incubated	
14	at 37°C for 1 hour, then placed on ice.	

15

16    **(b) Second strand synthesis.**

17

18    The following reagents were added to the first strand  
19    reaction:

20	DEPC-treated water	93 $\mu$ l
21	5x second strand buffer	30 $\mu$ l
22	10mM dNTPs	3 $\mu$ l
23	10u/ $\mu$ l <i>E. coli</i> DNA ligase	1 $\mu$ l
24	10u/ $\mu$ l <i>E. coli</i> DNA polymerase	4 $\mu$ l
25	2u/ $\mu$ l <i>E. coli</i> RNase H	1 $\mu$ l
26	The reaction was vortex mixed and incubated at 16°C for	
27	2 hours. 2 $\mu$ l (10u) of T4 DNA polymerase was added and	
28	incubation continued at 16°C for 5 minutes. The reaction	
29	was placed on ice and 10 $\mu$ l 0.5M EDTA added, then	
30	phenol-chloroform extracted, precipitated and vacuum	
31	dried.	

1

2 (c) Sal I adaptor ligation.

3

4 The cDNA pellet was resuspended in 25 $\mu$ l DEPC-treated  
5 water, and ligation set up as follows.

6	cDNA	25µl
7	5x T4 DNA ligase buffer	10µl
8	1µg/µl <i>Sal</i> I adapters*	10µl
9	1u/µl T4 DNA ligase	5µl

10 \*Sal I adapters: TCGACCCACGCGTCCG-3' (SEQ ID No 22)  
11 GGGTGCCGAGGC-5' (SEQ ID No 23)

12 The ligation was mixed gently and incubated for 16  
13 hours at 16°C, then phenol-chloroform extracted,  
14 precipitated and vacuum dried. The cDNA/adaptor pellet  
15 was resuspended in 41µl of DEPC-treated water and  
16 digested with 60 units of NotI at 37°C for 2 hours, then  
17 phenol-chloroform extracted, precipitated and vacuum  
18 dried. The cDNA pellet was re-dissolved in 100µl TEN  
19 buffer (10mM Tris pH 7.5, 0.1mM EDTA, 25mM NaCl) and  
20 size fractionated using a Sephadex S-500 HR column to  
21 remove unligated adapters and small cDNA fragments  
22 (<400bp) according to the manufacturers instructions.  
23 Fractions were checked by agarose gel electrophoresis  
24 and fractions containing cDNA less than 400 base pairs  
25 discarded, while the remaining fractions were pooled.

26

27 (d) PCR amplification of estrogen receptor DNA binding  
28 domain.

29

30 The estrogen receptor was PCR amplified from 5µl (150-  
31 250ng) of bone marrow cDNA using 25pmol of each of the  
PH2 140437v1 09/27/02 3:35 PM 40544.00101

1 primers pDM12FOR (SEQ ID No 24) (5'-  
2 AAAAGAATTCTGAATGTGTTATTTAGCTCAGGTCACTCTGACCTGATTATCAAG  
3 ACCCCACTTCACCCCCCT) and pDM12BAK (SEQ ID No 25) (5'-  
4 AAAAGCGGCCGCAGGGGGAGGAGGGTCCATGGAATCTGCCAAGGAG-3') in  
5 two 50µl reactions containing 0.1mM dNTPs, 2.5 units  
6 Taq DNA polymerase, and 1x PCR reaction buffer (10mM  
7 Tris-HCl pH 9.0, 5mM KCl, 0.01% Triton X®-100, 1.5mM  
8 MgCl<sub>2</sub>) (Promega Ltd, Southampton, U.K.). The pDM12FOR  
9 primer anneals to the 3'-end of the DNA binding domain  
10 of the estrogen receptor and incorporates two stop  
11 codons, the 38 base pair consensus estrogen receptor  
12 HRE sequence, and an EcoRI restriction site. The  
13 pDM12BAK primer anneals to the 5'-end of the DNA  
14 binding domain of the estrogen receptor and  
15 incorporates the (Gly)<sub>4</sub>Ser linker and the NotI  
16 restriction site.

17  
18 Reactions were overlaid with mineral oil and PCR  
19 carried out on a Techne PHC-3 thermal cycler for 30  
20 cycles of 94°C, 1 minute; 65°C, 1 minute; 72°C, 1  
21 minute. Reaction products were electrophoresed on an  
22 agarose gel, excised and products purified from the gel  
23 using a Geneclean II kit according to the manufacturers  
24 instructions (Biol01, La Jolla, California, U.S.A.).

25

26 **(e) Restriction digestion and ligation.**

27

28 The PCR reaction appended NotI and EcoRI restriction  
29 sites, the (Gly)<sub>4</sub>Ser linker, stop codons and the 38 base  
30 pair estrogen receptor target HRE nucleotide sequence  
31 to the estrogen receptor DNA binding domain sequence

1 (see Figure 1). The DNA PCR fragment and the target  
2 pDM6 vector (approximately 500ng) were NotI and EcoRI  
3 digested for 1 hour at 37°C, and DNA purified by agarose  
4 gel electrophoresis and extraction with Geneclean II  
5 kit (Bio101, La Jolla, California, U.S.A.). The  
6 estrogen receptor DNA binding domain cassette was  
7 ligated into the NotI-EcoRI digested pDM6 vector  
8 overnight at 16°C, phenol/chloroform extracted and  
9 precipitated then electroporated into TG1 *E. coli*  
10 (genotype: K12, ( $\Delta$ lac-pro), supE, thi, hsD5/F' traD36,  
11 proA<sup>+</sup>B<sup>+</sup>, LacI<sup>q</sup>, LacZ $\Delta$ 15) and plated onto 2xTY agar  
12 plates supplemented with 1% glucose and 100 $\mu$ g/ml  
13 ampicillin. Colonies were allowed to grow overnight at  
14 37°C. Individual colonies were picked into 5ml 2xTY  
15 supplemented with 1% glucose and 100 $\mu$ g/ml ampicillin  
16 and grown overnight at 37°C. Double stranded phagemid  
17 DNA was isolated with a Wizard SV plasmid DNA isolation  
18 kit and the sequence confirmed with a Prism dyedeoxy  
19 cycle sequencing kit (Perkin-Elmer, Warrington,  
20 Lancashire, U.K.) using M13FOR (SEQ ID No 26) (5'-  
21 GTAAAACGACGCCAGT) and M13REV (SEQ ID No 27) (5'-  
22 GGATAACAATTTCACACAGG) oligonucleotides. The pDM12 PDCP  
23 display vector DNA sequence between the HindIII and  
24 EcoRI restriction sites is shown in Figure 1.

25  
26 **Example 2. Insertion of a random-primed human**  
27 **lymphocyte cDNA into pDM12 and preparation of a master**  
28 **PDCP stock.**

29

1 Libraries of peptides can be constructed by many  
2 methods known to those skilled in the art. The example  
3 given describes a method for constructing a peptide  
4 library from randomly primed cDNA, prepared from mRNA  
5 isolated from a partially purified cell population.

6

7 mRNA was isolated from approximately  $10^9$  human  
8 peripheral blood lymphocytes using a polyATract 1000  
9 mRNA isolation kit (Promega, Southampton, UK). The cell  
10 pellet was resuspended in 4ml extraction buffer (4M  
11 guanidine thiocyanate, 25mM sodium citrate pH 7.1, 2%  
12  $\beta$ -mercaptoethanol). 8ml of pre-heated (70°C) dilution  
13 buffer (6xSSC, 10mM Tris pH 7.4, 1mM EDTA, 0.25% SDS,  
14 1%  $\beta$ -mercaptoethanol) was added to the homogenate and  
15 mixed thoroughly by inversion. 10 $\mu$ l of biotinylated  
16 oligo-dT (50 pmol/ $\mu$ l) was added, mixed and the mixture  
17 incubated at 70°C for 5 minutes. The lymphocyte cell  
18 lysate was transferred to 6x 2ml sterile tubes and spun  
19 at 13,000 rpm in a microcentrifuge for ten minutes at  
20 ambient temperature to produce a cleared lysate. During  
21 this centrifugation, streptavidin coated magnetic beads  
22 were resuspended and 6ml transferred to a sterile 50ml  
23 Falcon tube, then placed in the magnetic stand in a  
24 horizontal position until all the beads were captured.  
25 The supernatant was carefully poured off and beads  
26 resuspended in 6ml 0.5xSSC, then the capture repeated.  
27 This wash was repeated 3 times, and beads resuspended  
28 in a final volume of 6ml 0.5xSSC. The cleared lysate  
29 was added to the washed beads, mixed by inversion and  
30 incubated at ambient temperature for 2 minutes, then  
31 beads captured in the magnetic stand in a horizontal

1 position. The beads were resuspended gently in 2ml  
2 0.5xSSC and transferred to a sterile 2ml screwtop tube,  
3 then captured again in the vertical position, and the  
4 wash solution discarded. This wash was repeated twice  
5 more. 1ml of DEPC-treated water was added to the beads  
6 and mixed gently. The beads were again captured and the  
7 eluted mRNA transferred to a sterile tube. 50 $\mu$ l was  
8 electrophoresed to check the quality and quantity of  
9 mRNA, while the remainder was precipitated with 0.1  
10 volumes 3M sodium acetate and three volumes absolute  
11 ethanol at -80°C overnight in 4 aliquots in sterile  
12 1.5ml screwtop tubes.

13

14 Double stranded cDNA was synthesised as described in  
15 Example 1 using 5 $\mu$ g of lymphocyte mRNA as template.  
16 The cDNA was PCR amplified using oligonucleotides  
17 CDNAPCRFOR (SEQ ID No 28) 5'-  
18 AAAGCGGCCGCACTGGCCTGAGAGA), which anneals to the cDNA  
19 synthesis oligonucleotide described in Example 1 which  
20 is present at the 3'-end of all synthesised cDNA  
21 molecules incorporates a NotI restriction site, and an  
22 equimolar mixture of CDNAPCRBAK1, CDNAPCRBAK2 and  
23 CDNAPCRBAK3.

24 CDNAPCRBAK1: (SEQ ID No 29) 5'-

25 AAAAGGCCAGCCGGCCATGGCCCAGCCCCACCACGCGTCCG,

26 CDNAPCRBAK2: (SEQ ID No 30) 5'-

27 AAAAGGCCAGCCGGCCATGGCCCAGTCCCACCACGCGTCCG,

28 CDNAPCRBAK3: (SEQ ID No 31) 5'-

29 AAAAGGCCAGCCGGCCATGGCCCAGTACCCACCACGCGTCCG),

30 all three of which anneal to the SalI adaptor sequence  
31 found at the 5'-end of the cDNA and incorporate a SfiI

1 restriction site at the cDNA 5'-end. Ten PCR reactions  
2 were carried out using 2 $\mu$ l of cDNA (50ng) per reaction  
3 as described in Example 1 using 25 cycles of 94°C, 1  
4 minute; 60°C, 1 minute; 72°C, 2 minutes. The reactions  
5 were pooled and a 20 $\mu$ l aliquot checked by agarose gel  
6 electrophoresis, the remainder was phenol/chloroform  
7 extracted and ethanol precipitated and resuspended in  
8 100 $\mu$ l sterile water. 5 $\mu$ g of pDM12 vector DNA and  
9 lymphocyte cDNA PCR product were SfiI-NotI digested  
10 phenol/chloroform extracted and small DNA fragments  
11 removed by size selection on Chromaspin 1000 spin  
12 columns (Clontech, Palo Alto, California, U.S.A.) by  
13 centrifugation at 700g for 2 minutes at room  
14 temperature. Digested pDM12 and lymphocyte cDNA were  
15 ethanol precipitated and ligated together for 16 hours  
16 at 16°C. The ligated DNA was precipitated and  
17 electroporated in to TG1 *E. coli*. Cells were grown in  
18 1ml SOC medium per cuvette used for 1 hour at 37°C, and  
19 plated onto 2xTY agar plates supplemented with 1%  
20 glucose and 100 $\mu$ g/ml ampicillin. 10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup>  
21 dilutions of the electroporated bacteria were also  
22 plated to assess library size. Colonies were allowed to  
23 grow overnight at 30°C. 2x10<sup>8</sup> ampicillin resistant  
24 colonies were recovered on the agar plates.  
25 The bacteria were then scraped off the plates into 40ml  
26 2xTY broth supplemented with 20% glycerol, 1% glucose  
27 and 100 $\mu$ g/ml ampicillin. 5ml was added to a 20ml 2xTY  
28 culture broth supplemented with 1% glucose and 100 $\mu$ g/ml  
29 ampicillin and infected with 10<sup>11</sup> kanamycin resistance  
30 units (kru) M13K07 helper phage at 37°C for 30 minutes

1 without shaking, then for 30 minutes with shaking at  
2 200rpm. Infected bacteria were transferred to 200ml  
3 2xTY broth supplemented with 25 $\mu$ g/ml kanamycin,  
4 100 $\mu$ g/ml ampicillin, and 20 $\mu$ M IPTG, then incubated  
5 overnight at 37°C, shaking at 200rpm. Bacteria were  
6 pelleted at 4000rpm for 20 minutes in 50ml Falcon  
7 tubes, and 40ml 2.5M NaCl/20% PEG 6000 was added to  
8 200ml of particle supernatant, mixed vigorously and  
9 incubated on ice for 1 hour to precipitate PDCP  
10 particles. Particles were pelleted at 11000rpm for 30  
11 minutes in 250ml Oakridge tubes at 4°C in a Sorvall RC5B  
12 centrifuge, then resuspended in 2ml PBS buffer after  
13 removing all traces of PEG/NaCl with a pipette, then  
14 bacterial debris removed by a 5 minute 13500rpm spin in  
15 a microcentrifuge. The supernatent was filtered through  
16 a 0.45 $\mu$ m polysulfone syringe filter and stored at -20°C.  
17

18 **Example 3. Isolation of human immunoglobulin kappa  
19 light chains by repeated rounds of selection against  
20 anti-human kappa antibody.**

21  
22 For the first round of library selection a 70x11mm NUNC  
23 Maxisorp Immunotube (Life Technologies, Paisley,  
24 Scotland U.K.) was coated with 2.5ml of 10 $\mu$ g/ml of  
25 anti-human kappa antibody (Seralab, Crawley Down,  
26 Sussex, U.K.) in PBS for 2 hours at 37°C. The tube was  
27 rinsed three times with PBS (fill & empty) and blocked  
28 with 3ml PBS/2% BSA for 2 hours at 37°C and washed as  
29 before. 4x10<sup>12</sup> a.r.u. of pDM12-lymphocyte cDNA PDCP  
30 stock was added in 2ml 2% BSA/PBS/0.05% Tween 20, and

1 incubated for 30 minutes on a blood mixer, then for 90  
2 minutes standing at ambient temperature. The tube was  
3 washed ten times with PBS/0.1% Tween 20, then a further  
4 ten times with PBS only. Bound particles were eluted in  
5 1ml of freshly prepared 0.1M triethylamine for 10  
6 minutes at ambient temperature on a blood mixer. Eluted  
7 particles were transferred to 0.5ml 1M Tris pH 7.4,  
8 vortex mixed briefly and transferred to ice.

9

10 Neutralised particles were added to 10ml log phase TG1  
11 E coli bacteria (optical density: OD<sub>600nm</sub> 0.3-0.5) and  
12 incubated at 37°C without shaking for 30 minutes, then  
13 with shaking at 200rpm for 30 minutes. 10<sup>-3</sup>, 10<sup>-4</sup> & 10<sup>-5</sup>  
14 dilutions of the infected culture were prepared to  
15 estimate the number of particles recovered, and the  
16 remainder was spun at 4000 rpm for 10 minutes, and the  
17 pellet resuspended in 300µl 2xTY medium by vortex  
18 mixing. Bacteria were plated onto 2xTY agar plates  
19 supplemented with 1% glucose and 100µg/ml ampicillin.  
20 Colonies were allowed to grow overnight at 30°C.

21

22 A PDCP stock was prepared from the bacteria recovered  
23 from the first round of selection, as described in  
24 Example 2 from a 100ml overnight culture. 250µl of the  
25 round 1 amplified PDCP stock was then selected against  
26 anti-human kappa antibody as described above with the  
27 tube was washed twelve times with PBS/0.1% Tween 20,  
28 then a further twelve times with PBS only.

29

30 To identify selected clones, eighty-eight individual  
31 clones recovered from the second round of selection

1 were then tested by ELISA for binding to anti-human  
2 kappa antibody. Individual colonies were picked into  
3 100 $\mu$ l 2xTY supplemented with 100 $\mu$ g/ml ampicillin and 1%  
4 glucose in 96-well plates (Costar) and incubated at 37°C  
5 and shaken at 200rpm for 4 hours. 25 $\mu$ l of each culture  
6 was transferred to a fresh 96-well plate, containing  
7 25 $\mu$ l/well of the same medium plus 10<sup>7</sup> k.r.u. M13K07  
8 kanamycin resistant helper phage and incubated at 37°C  
9 for 30 minutes without shaking, then incubated at 37°C  
10 and shaken at 200rpm for a further 30 minutes. 160 $\mu$ l of  
11 2xTY supplemented with 100 $\mu$ g/ml ampicillin, 25 $\mu$ g/ml  
12 kanamycin, and 20 $\mu$ M IPTG was added to each well and  
13 particle amplification continued for 16 hours at 37°C  
14 while shaking at 200rpm. Bacterial cultures were spun  
15 in microtitre plate carriers at 2000g for 10 minutes at  
16 4°C in a benchtop centrifuge to pellet bacteria and  
17 culture supernatant used for ELISA.

18

19 A Dynatech Immulon 4 ELISA plate was coated with  
20 200ng/well anti-human kappa antibody in 100 $\mu$ l /well PBS  
21 for one hour at 37°C. The plate was washed 2x200 $\mu$ l/well  
22 PBS and blocked for 1 hour at 37°C with 200 $\mu$ l/well 2%  
23 BSA/PBS and then washed 2x200 $\mu$ l/well PBS. 50 $\mu$ l PDCP  
24 culture supernatant was added to each well containing  
25 50 $\mu$ l/well 4% BSA/PBS/0.1%Tween 20, and allowed to bind  
26 for 1 hour at ambient temperature. The plate was washed  
27 three times with 200 $\mu$ l/well PBS/0.1% Tween 20, then  
28 three times with 200 $\mu$ l/well PBS. Bound PDCPs were  
29 detected with 100 $\mu$ l/well, 1:5000 diluted anti-M13-HRP  
30 conjugate (Pharmacia) in 2% BSA/PBS/0.05% Tween 20 for

1   1 hour at ambient temperature and the plate washed six  
2   times as above. The plate was developed for 5 minutes  
3   at ambient temperature with 100µl/well freshly prepared  
4   TMB (3,3',5,5'-Tetramethylbenzidine) substrate buffer  
5   (0.005% H<sub>2</sub>O<sub>2</sub>, 0.1mg/ml TMB in 24mM citric acid/52mM  
6   sodium phosphate buffer pH 5.2). The reaction was  
7   stopped with 100µl/well 12.5% H<sub>2</sub>SO<sub>4</sub> and read at 450nm.  
8   (ELISA data for binding clones is shown in Figure 2).

9

10 These clones were then sequenced with M13REV primer  
11 (SEQ ID No 27) as in Example 1. The sequence of two of  
12 the clones isolated is shown in Figure 3 (see SEQ ID  
13 Nos 7 to 10).

14

15 **Example 4. Construction of the pDM14 N-terminal display  
16 vector**

17

18 It would be useful to design vectors that contain a  
19 second DBD binding sequence, such as a second estrogen  
20 receptor HRE sequence, thus allowing the display of  
21 increased numbers of peptides per PDCP. Peale et al.  
22 (1988, Proc. Natl. Acad. Sci. USA 85: 1038-1042)  
23 describe a number of estrogen receptor HRE sequences.  
24 These sequences were used to define an HRE sequence,  
25 which differs from that cloned in pDM12, which we used  
26 to create a second N-terminal display vector (pDM14).  
27 The oligonucleotide: 5'-AAAAGAATTGAGGTTACATTAACCTTGTT  
28 CCGGTCACTGACCCAAGTCGACCTGAATGTGTTATTTAG-3' (SEQ ID  
29 No 32) was synthesised and used to mutagenise pDM12 by  
30 PCR with pDM12BAK oligonucleotide as described in  
31 Example 1 using 100ng pDM12 vector DNA as template. The

1 resulting DNA fragment, which contained the estrogen  
2 receptor DBD and two HRE sequences separated by a SalI  
3 restriction enzyme site, was NotI-EcoRI restriction  
4 enzyme digested and cloned into NotI-EcoRI digested  
5 pDM12 vector DNA as described in Example 1 to create  
6 pDM14. The sequence of pDM14 between the HindIII and  
7 EcoRI restriction enzyme sites was checked by DNA  
8 sequencing. The final vector sequence between these two  
9 sites is shown in Figure 4 (see SEQ ID Nos 11 and 12).

10

11 **Example 5. Construction of the pDM16 C-terminal display**  
12 **vector**

13

14 In order to demonstrate the display of peptides fused  
15 to the C-terminus of a DBD on a PDCP a suitable vector,  
16 pDM16, was created.

17

18 In pDM16 the pelB leader DNA sequence is fused directly  
19 to the estrogen receptor DBD sequence removing the  
20 multiple cloning sites and the Gly<sub>4</sub>Ser linker DNA  
21 sequence found in pDM12 and pDM14, which are appended  
22 to the C-terminal end of the DBD sequence upstream of  
23 the HRE DNA sequence.

24

25 To create this vector two separate PCR reactions were  
26 carried out on a Techne Progene thermal cycler for 30  
27 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1  
28 minute. Reaction products were electrophoresed on an  
29 agarose gel, excised and products purified from the gel  
30 using a Mermaid or Geneclean II kit, respectively,

1 according to the manufacturers instructions (Bio101, La  
2 Jolla, California, U.S.A.).

3

4 In the first, the 5'-untranslated region and pelB  
5 leader DNA sequence was amplified from 100ng of pDM12  
6 vector DNA using 50pmol of each of the oligonucleotides  
7 pelBFOR (SEQ ID No 33) (5'-CCTTGGCAGATTCCATCT  
8 CGGCCATTGCCGGC-3') and M13REV (SEQ ID NO 27) (see  
9 above) in a 100 $\mu$ l reaction containing 0.1mM dNTPs, 2.5  
10 units Taqplus DNA polymerase, and 1x High Salt PCR  
11 reaction buffer (20mM Tris-HCl pH 9.2, 60mM KCl, 2mM  
12 MgCl<sub>2</sub>) (Stratagene Ltd, Cambridge, U.K.).

13

14 In the second, the 3'-end of the pelB leader sequence  
15 and the estrogen receptor DBD was amplified from 100ng  
16 of pDM12 vector DNA using 50pmol of each of the  
17 oligonucleotides pelBAK (SEQ ID No 34) (5'-CCGGCAA  
18 TGGCCGAGATGGAATCTGCCAAGG-3') and pDM16FOR (SEQ ID No  
19 35) (5'-TTTGTGACTCAATCAGTTATGCGGCCAGCTGCAGG  
20 AGGGCCGGCTGGGCCGACCCCTCCTCCCCAGACCCCACCC-3') in a  
21 100 $\mu$ l reaction containing 0.1mM dNTPs, 2.5 units  
22 Taqplus DNA polymerase, and 1x High Salt PCR reaction  
23 buffer (Stratagene Ltd, Cambridge, U.K.). Following gel  
24 purification both products were mixed together and a  
25 final round of PCR amplification carried out to link  
26 the two products together as described above, in a  
27 100 $\mu$ l reaction containing 0.1mM dNTPs, 2.5 units Taq  
28 DNA polymerase, and 1x PCR reaction buffer (10mM Tris-  
29 HCl pH 9.0, 5mM KCl, 0.01% Triton X<sup>®</sup>-100, 1.5mM MgCl<sub>2</sub>)  
30 (Promega Ltd, Southampton, U.K.).

31

1 The resulting DNA fragment, was HindIII-SalI  
2 restriction enzyme digested and cloned into HindIII-  
3 SalI digested pDM14 vector DNA as described in Example  
4 1 to create pDM16. The sequence of pDM16 between the  
5 HindIII and EcoRI restriction enzyme sites was checked  
6 by DNA sequencing. The final vector sequence between  
7 these two sites is shown in Figure 5 (see SEQ ID Nos 13  
8 and 14).

9

10 **Example 6. Display of the C-terminal fragment of human  
11 N-cadherin on the surface of a PDCP**

12

13 cDNA libraries of peptides can be constructed by many  
14 methods known to those skilled in the art. One commonly  
15 used method for constructing a peptide library uses  
16 oligo dT primed cDNA, prepared from polyA+ mRNA. In  
17 this method the first-strand synthesis is carried out  
18 using an oligonucleotide which anneals to the 3'-end  
19 polyA tail of the mRNA composed of T<sub>n</sub> (where n is  
20 normally between 10 and 20 bases) and a restriction  
21 enzyme site such as NotI to facilitate cloning of cDNA.  
22 The cDNA cloned by this method is normally composed of  
23 the polyA tail, the 3'- end untranslated region and the  
24 C-terminal coding region of the protein. As an example  
25 of the C-terminal display of peptides on a PDCP, a  
26 human cDNA isolated from a library constructed by the  
27 above method was chosen.

28

29 The protein N-cadherin is a cell surface molecule  
30 involved in cell-cell adhesion. The C-terminal  
31 cytoplasmic domain of the human protein (Genbank

1 database accession number: M34064) is recognised by a  
2 commercially available monoclonal antibody which was  
3 raised against the C-terminal 23 amino acids of chicken  
4 N-cadherin (SIGMA catalogue number: C-1821). The 1.4kb  
5 human cDNA fragment encoding the C-terminal 99 amino  
6 acids, 3'- untranslated region and polyA tail (NotI  
7 site present at the 3'-end of the polyA tail) was  
8 amplified from approximately 20ng pDM7-NCAD#C with  
9 25pmol of each oligonucleotide M13FOR (SEQ ID No 26)  
10 and CDNPCRBAK1 (SEQ ID No 29) (see above) in a 50µl  
11 reaction containing 0.1mM dNTPs, 2.5 units Taqplus DNA  
12 polymerase, and 1x High Salt PCR reaction buffer (20mM  
13 Tris-HCl pH 9.2, 60mM KCl, 2mM MgCl<sub>2</sub>) (Stratagene Ltd,  
14 Cambridge, U.K.) on a Techne Progene thermal cycler for  
15 30 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1  
16 minute. Following gel purification and digestion with  
17 SfiI and NotI restriction enzymes, the PCR product was  
18 cloned into pDM16 using an analogous protocol as  
19 described in Example 1.

20  
21 Clones containing inserts were identified by ELISA of  
22 96 individual PDCP cultures prepared as described in  
23 Example 3. A Dynatech Immulon 4 ELISA plate was coated  
24 with 1:250 diluted anti-pan cadherin monoclonal  
25 antibody in 100µl /well PBS overnight at 4°C. The plate  
26 was washed 3x200µl/well PBS and blocked for 1 hour at  
27 37°C with 200µl/well 2% Marvel non-fat milk powder/PBS  
28 and then washed 2x200µl/well PBS. 50µl PDCP culture  
29 supernatant was added to each well containing 50µl/well  
30 4% Marvel/PBS, and allowed to bind for 1 hour at  
31 ambient temperature. The plate was washed three times

1 with 200 $\mu$ l/well PBS/0.1% Tween 20, then three times  
2 with 200 $\mu$ l/well PBS. Bound PDCPs were detected with  
3 100 $\mu$ l/well, 1:5000 diluted anti-M13-HRP conjugate  
4 (Pharmacia) in 2% Marvel/PBS for 1 hour at ambient  
5 temperature and the plate washed six times as above.  
6 The plate was developed for 15 minutes at ambient  
7 temperature with 100 $\mu$ l/well freshly prepared TMB  
8 (3,3',5,5'-Tetramethylbenzidine) substrate buffer  
9 (0.005% H<sub>2</sub>O<sub>2</sub>, 0.1mg/ml TMB in 24mM citric acid/52mM  
10 sodium phosphate buffer pH 5.2). The reaction was  
11 stopped with 100 $\mu$ l/well 12.5% H<sub>2</sub>SO<sub>4</sub> and read at 450nm.  
12 The nucleotide sequence of an ELISA positive clone  
13 insert and DBD junction was checked by DNA sequencing  
14 using oligonucleotides M13FOR (SEQ ID No 26) (see  
15 Example 1) and ORSEQBAK (SEQ ID No 36) (5'-  
16 TGTTGAAACACAAGGCCAG-3').  
17  
18 A fifty-fold concentrated stock of C-terminal N-  
19 cadherin PDCP particles was prepared by growing the un-  
20 infected TG1 clone in 1ml 2xTY culture broth  
21 supplemented with 1% glucose and 100 $\mu$ g/ml ampicillin  
22 for five hours at 37°C, shaking at 200rpm and infecting  
23 with 10<sup>8</sup> kanamycin resistance units (kru) M13K07 helper  
24 phage at 37°C for 30 minutes without shaking, then for  
25 30 minutes with shaking at 200rpm. Infected bacteria  
26 were transferred to 20ml 2xTY broth supplemented with  
27 25 $\mu$ g/ml kanamycin, 100 $\mu$ g/ml ampicillin, and 20 $\mu$ M IPTG,  
28 then incubated overnight at 30°C, shaking at 200rpm.  
29 Bacteria were pelleted at 4000rpm for 20 minutes in  
30 50ml Falcon tubes, and 4ml 2.5M NaCl/20% PEG 6000 was

1 added to 20ml of PDCP supernatant, mixed vigorously and  
2 incubated on ice for 1 hour to precipitate particles.

3

4 The particles were pelleted at 11000rpm for 30 minutes  
5 in 50ml Oakridge tubes at 4°C in a Sorvall RC5B  
6 centrifuge, then resuspended in PBS buffer after  
7 removing all traces of PEG/NaCl with a pipette, then  
8 bacterial debris removed by a 5 minute 13500rpm spin in  
9 a microcentrifuge. The supernatant was filtered through  
10 a 0.45µm polysulfone syringe filter. The concentrated  
11 stock was two-fold serially diluted and used in ELISA  
12 against plates coated with anti-pan-cadherin antibody  
13 as described above (see Figure 6).

14

15 This example demonstrates the principle of C-terminal  
16 display using PDCPs, that C-terminal DBD-peptide fusion  
17 PDCPs can be made which can be detected in ELISA, and  
18 the possibility that oligo dT primed cDNA libraries may  
19 be displayed using this method.

20

21 **Example 7. Display of *in vivo* biotinylated C-terminal**  
22 **domain of human propionyl CoA carboxylase on the**  
23 **surface of a PDCP**

24

25 Example 6 shows that the C-terminal domain of human N-  
26 cadherin can be expressed on the surface of a PDCP as a  
27 C-terminal fusion with the DBD. Here it is shown that  
28 the C-terminal domain of another human protein  
29 propionyl CoA carboxylase alpha chain (Genbank  
30 accession number: X14608) can similarly be displayed,  
31 suggesting that this methodology may be general.

1  
2 The alpha sub-unit of propionyl CoA carboxylase alpha  
3 chain (PCC) contains 703 amino acids and is normally  
4 biotinylated at position 669. It is demonstrated that  
5 the PCC peptide displayed on the PDCP is biotinylated,  
6 as has been shown to occur when the protein is  
7 expressed in bacterial cells (Leon-Del-Rio & Gravel;  
8 1994, J. Biol. Chem. 37, 22964-22968).

9

10 The 0.8kb human cDNA fragment of PCC alpha encoding the  
11 C-terminal 95 amino acids, 3'- untranslated region and  
12 polyA tail (NotI site present at the 3'-end of the  
13 polyA tail) was amplified and cloned into pDM16 from  
14 approximately 20ng pDM7-PCC#C with 25pmol of each  
15 oligonucleotide M13FOR (SEQ ID No 26) and CDNPCRBAK1  
16 (SEQ ID No 29) as described in Example 6.

17

18 Clones containing inserts were identified by ELISA as  
19 described in Example 6, except that streptavidin was  
20 coated onto the ELISA plate at 250ng/well, in place of  
21 the anti-cadherin antibody. The nucleotide sequence of  
22 an ELISA positive clone insert and DBD junction was  
23 checked by DNA sequencing using oligonucleotides M13FOR  
24 (SEQ ID No 26) and ORSEQBAK (SEQ ID No 36) (see above).  
25 A fifty-fold concentrated stock of C-terminal PCC PDCP  
26 particles was prepared and tested in ELISA against  
27 streptavidin as described in Example 6 (see Figure 7).

28

29 This example shows not only that the peptide can be  
30 displayed as a C-terminal fusion on a PDCP, but also  
31 that *in vivo* modified peptides can be displayed.

1

2   **Example 8. Construction of a human scFv PDCP display**  
3   **library**

4

5   This example describes the generation of a human  
6   antibody library of scFvs made from an un-immunised  
7   human. The overall strategy for the PCR assembly of  
8   scFv fragments is similar to that employed by Marks, J.  
9   D. et al. 1991, J. Mol. Biol. 222: 581-597. The  
10   antibody gene oligonucleotides used to construct the  
11   library are derived from the Marke et al., paper and  
12   from sequence data extracted from the Kabat database  
13   (Kabat, E. A. et al., Sequences of Proteins of  
14   Immunological Interest. 4<sup>th</sup> edition. U.S. Department of  
15   Health and Human Services. 1987). The three linker  
16   oligonucleotides are described by Zhou et al. (1994,  
17   Nucleic Acids Res., 22: 888-889), all oligonucleotides  
18   used are detailed in Table 1.

19

20   First, mRNA was isolated from peripheral blood  
21   lymphocytes and cDNA prepared for four repertoires of  
22   antibody genes IgD, IgM, Igκ and Igλ, using four  
23   separate cDNA synthesis primers. VH genes were  
24   amplified from IgD and IgM primed cDNA, and VL genes  
25   were amplified from Igκ and Igλ primed cDNA. A portion  
26   of each set of amplified heavy chain or light chain DNA  
27   was then spliced with a separate piece of linker DNA  
28   encoding the 15 amino acids (Gly<sub>4</sub> Ser)<sub>3</sub> (Huston, J. S.  
29   et al. 1989, Gene, 77: 61). The 3'-end of the VH PCR  
30   products and the 5'-end of the VL PCR products overlap  
31   the linker sequence as a result of incorporating linker

1 sequence in the JH, V $\kappa$  and V $\lambda$  family primer sets (Table  
2 1). Each VH-linker or linker-VL DNA product was then  
3 spliced with either VH or VL DNA to produce the primary  
4 scFv product in a VH-linker-VL configuration. This scFv  
5 product was then amplified and cloned into pDM12 as a  
6 SfiI-NotI fragment, electroporated into TG1 and a  
7 concentrated PDCP stock prepared.

8

9 **mRNA isolation and cDNA synthesis.**

10 Human lymphocyte mRNA was purified as described in  
11 Example 2. Separate cDNA reactions were performed with  
12 IGDCDNAFOR (SEQ ID No 37), IGMCDNAFOR (SEQ ID No 38),  
13 IGKCDNAFOR (SEQ ID No 39) and IG $\lambda$ CDNAFOR (SEQ ID No 40)  
14 oligonucleotides. 50pmol of each primer was added to  
15 approximately 5 $\mu$ g of mRNA in 20 $\mu$ l of nuclease free  
16 water and heated to 70°C for 5 minutes and cooled  
17 rapidly on ice, then made up to a final reaction volume  
18 of 100 $\mu$ l containing 50mM Tris pH 8.3, 75mM KCl, 3mM  
19 MgCl<sub>2</sub>, 10mM DTT, 0.5mM dNTPs, and 2000 units of  
20 Superscript II reverse transcriptase (Life  
21 Technologies, Paisley, Scotland, U.K.). The reactions  
22 were incubated at 37°C for two hours, then heated to  
23 95°C for 5 minutes.

24

25 **Primary PCRs.**

26 For the primary PCR amplifications separate  
27 amplifications were set up for each family specific  
28 primer with either an equimolar mixture of the JHFOR  
29 primer set (SEQ ID Nos 41 to 44) for IgM and IgD cDNA,  
30 or with SCFV $\kappa$ FOR (SEQ ID No 51) or SCFV $\lambda$ FOR (SEQ ID No

1       52) for IgK or Ig $\lambda$  cDNA respectively e.g. VH1BAK and  
2       JHFOR set; V $\kappa$ 2BAK (SEQ ID No 54) and SCFV $\kappa$ FOR (SEQ ID  
3       No 51); V $\lambda$ 3aBAK (SEQ ID No 66) and SCFV $\lambda$ FOR (SEQ ID No  
4       52) etc. Thus, for IgM, IgD and IgK cDNA six separate  
5       reactions were set up, and seven for Ig $\lambda$  cDNA. A 50 $\mu$ l  
6       reaction mixture was prepared containing 2 $\mu$ l cDNA,  
7       25pmol of the appropriate FOR and BAK primers, 0.1mM  
8       dNTPs, 2.5 units Taqplus DNA polymerase, and 1x High  
9       Salt PCR reaction buffer (20mM Tris-HCl pH 9.2, 60mM  
10      KCl, 2mM MgCl<sub>2</sub>) (Stratagene Ltd, Cambridge, U.K.).  
11      Reactions were amplified on a Techne Progene thermal  
12      cycler for 30 cycles of 94°C, 1 minute; 60°C, 1 minute;  
13      72°C, 2 minutes, followed by 10 minutes at 72°C. Fifty  
14      microlitres of all 25 reaction products were  
15      electrophoresed on an agarose gel, excised and products  
16      purified from the gel using a Geneclean II kit  
17      according to the manufacturers instructions (Biol01, La  
18      Jolla, California, U.S.A.). All sets of IgD, IgM, IgK  
19      or Ig $\lambda$  reaction products were pooled to produce VH or  
20      VL DNA sets for each of the four repertoires. These  
21      were then adjusted to approximately 20ng/ $\mu$ l.  
22

23      **Preparation of linker.**

24      Linker product was prepared from eight 100 $\mu$ l reactions  
25      containing 5ng LINKAMP3T (SEQ ID No 76) template  
26      oligonucleotide, 50pmol of LINKAMP3 (SEQ ID No 74) and  
27      LINKAMP5 (SEQ ID No 75) primers, 0.1mM dNTPs, 2.5 units  
28      Taqplus DNA polymerase, and 1x High Salt PCR reaction  
29      buffer (20mM Tris-HCl pH 9.2, 60mM KCl, 2mM MgCl<sub>2</sub>)  
30      (Stratagene Ltd, Cambridge, U.K.). Reactions were

1 amplified on a Techne Progene thermal cycler for 30  
2 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1  
3 minute, followed by 10 minutes at 72°C. All reaction  
4 product was electrophoresed on a 2% low melting point  
5 agarose gel, excised and products purified from the gel  
6 using a Mermaid kit according to the manufacturers  
7 instructions (Bio101, La Jolla, California, U.S.A.) and  
8 adjusted to 5ng/μl.

9

10 **First stage linking.**

11 Four linking reactions were prepared for each  
12 repertoire using 20ng of VH or VL DNA with 5ng of  
13 Linker DNA in 100μl reactions containing (for IgM or  
14 IgD VH) 50pmol of LINKAMPFOR and VH1-6BAK set, or,  
15 50pmol LINKAMPBAK and either SCFVκFOR (Igκ) or SCFVλFOR  
16 (Igλ), 0.1mM dNTPs, 2.5 units Taq DNA polymerase, and  
17 1x PCR reaction buffer (10mM Tris-HCl pH 9.0, 5mM KCl,  
18 0.01% Triton X®-100, 1.5mM MgCl<sub>2</sub>) (Promega Ltd,  
19 Southampton, U.K.). Reactions were amplified on a  
20 Techne Progene thermal cycler for 30 cycles of 94°C, 1  
21 minute; 60°C, 1 minute; 72°C, 2 minutes, followed by 10  
22 minutes at 72°C. Reaction products were electrophoresed  
23 on an agarose gel, excised and products purified from  
24 the gel using a Geneclean II kit according to the  
25 manufacturers instructions (Bio101, La Jolla,  
26 California, U.S.A.) and adjusted to 20ng/μl.

27

28 **Final linking and reamplification.**

29 To prepare the final scFv DNA products, five 100μl  
30 reactions were performed for VH-LINKER plus VL DNA,

1 and, five 100 $\mu$ l reactions were performed for VH plus  
2 LINKER-VL DNA for each of the four final repertoires  
3 (IgM VH-VK, VH-V $\lambda$ ; IgD VH-VK, VH-V $\lambda$ ) as described in  
4 step (d) above using 20ng of each component DNA as  
5 template. Reaction products were electrophoresed on an  
6 agarose gel, excised and products purified from the gel  
7 using a Geneclean II kit according to the manufacturers  
8 instructions (Biol01, La Jolla, California, U.S.A.) and  
9 adjusted to 20ng/ $\mu$ l. Each of the four repertoires was  
10 then re-amplified in a 100 $\mu$ l reaction volume containing  
11 2ng of each linked product, with 50pmol VHBAK1-6 (SEQ  
12 ID Nos 53 to 58) and either the JKFOR (SEQ ID Nos 66 to  
13 70) or J $\lambda$ FOR (SEQ ID Nos 71 to 73) primer sets, in the  
14 presence of 0.1mM dNTPs, 2.5 units Taq DNA polymerase,  
15 and 1x PCR reaction buffer (10mM Tris-HCl pH 9.0, 5mM  
16 KCl, 0.01% Triton X<sup>®</sup>-100, 1.5mM MgCl<sub>2</sub>) (Promega Ltd,  
17 Southampton, U.K.). Thirty reactions were performed per  
18 repertoire to generate enough DNA for cloning.  
19 Reactions were amplified on a Techne Progene thermal  
20 cycler for 25 cycles of 94°C, 1 minute; 65°C, 1 minute;  
21 72°C, 2 minutes, followed by 10 minutes at 72°C.  
22 Reaction products were phenol-chloroform extracted,  
23 ethanol precipitated, vacuum dried and re-suspended in  
24 80 $\mu$ l nuclease free water.

25

26 **Cloning into pDM12.**

27 Each of the four repertoires was SfiI-NotI digested,  
28 and electrophoresed on an agarose gel, excised and  
29 products purified from the gel using a Geneclean II kit  
30 according to the manufacturers instructions (Biol01, La

1 Jolla, California, U.S.A.). Each of the four  
2 repertoires was ligated overnight at 16°C in 140µl with  
3 10µg of SfiI-NotI cut pDM12 prepared as in Example 2,  
4 and 12 units of T4 DNA ligase (Life Technologies,  
5 Paisley, Scotland, U.K.). After incubation the  
6 ligation were adjusted to 200µl with nuclease free  
7 water, and DNA precipitated with 1µl 20mg/ml glycogen,  
8 100µl 7.5M ammonium acetate and 900µl ice-cold (-20°C)  
9 absolute ethanol, vortex mixed and spun at 13,000rpm  
10 for 20 minutes in a microfuge to pellet DNA. The  
11 pellets were washed with 500µl ice-cold 70% ethanol by  
12 centrifugation at 13,000rpm for 2 minutes, then vacuum  
13 dried and re-suspended in 10µl DEPC-treated water. 1µl  
14 aliquots of each repertoire was electroporated into  
15 80µl *E. coli* (TG1). Cells were grown in 1ml SOC medium  
16 per cuvette used for 1 hour at 37°C, and plated onto  
17 2xTY agar plates supplemented with 1% glucose and  
18 100µg/ml ampicillin. 10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup> dilutions of the  
19 electroporated bacteria were also plated to assess  
20 library size. Colonies were allowed to grow overnight  
21 at 30°C. Cloning into SfiI-NotI digested pDM12 yielded  
22 an IgM-κ/λ repertoire of 1.16x10<sup>9</sup> clones, and an IgD-κ/λ  
23 repertoire of 1.21x10<sup>9</sup> clones.

24

25 **Preparation of PDCP stock.**

26 Separate PDCP stocks were prepared for each repertoire  
27 library. The bacteria were then scraped off the plates  
28 into 30ml 2xTY broth supplemented with 20% glycerol, 1%  
29 glucose and 100µg/ml ampicillin. 3ml was added to a  
30 50ml 2xTY culture broth supplemented with 1% glucose  
PH2 140437v1 09/27/02 3:35 PM 40544.00101

1 and 100 $\mu$ g/ml ampicillin and infected with 10<sup>11</sup> kanamycin  
2 resistance units (kru) M13K07 helper phage at 37°C for  
3 30 minutes without shaking, then for 30 minutes with  
4 shaking at 200rpm. Infected bacteria were transferred  
5 to 500ml 2xTY broth supplemented with 25 $\mu$ g/ml  
6 kanamycin, 100 $\mu$ g/ml ampicillin, and 20 $\mu$ M IPTG, then  
7 incubated overnight at 30°C, shaking at 200rpm. Bacteria  
8 were pelleted at 4000rpm for 20 minutes in 50ml Falcon  
9 tubes, and 80ml 2.5M NaCl/20% PEG 6000 was added to  
10 400ml of particle supernatant, mixed vigorously and  
11 incubated on ice for 1 hour to precipitate PDCP  
12 particles. Particles were pelleted at 11000rpm for 30  
13 minutes in 250ml Oakridge tubes at 4°C in a Sorvall RC5B  
14 centrifuge, then resuspended in 40ml water and 8ml 2.5M  
15 NaCl/20% PEG 6000 added to reprecipitate particles,  
16 then incubated on ice for 20 minutes. Particles were  
17 again pelleted at 11000rpm for 30 minutes in 50ml  
18 Oakridge tubes at 4°C in a Sorvall RC5B centrifuge, then  
19 resuspended in 5ml PBS buffer, after removing all  
20 traces of PEG/NaCl with a pipette. Bacterial debris was  
21 removed by a 5 minute 13500rpm spin in a  
22 microcentrifuge. The supernatant was filtered through a  
23 0.45 $\mu$ m polysulfone syringe filter, adjusted to 20%  
24 glycerol and stored at -70°C.

25

26 **Example 9. Isolation of binding activity from a N-**  
27 **terminal display PDCP library of human scFvs**

28

29 The ability to select binding activities to a target of  
30 interest from a human antibody library is important due

1 to the possibility of generating therapeutic human  
2 antibodies. In addition, such libraries allow the  
3 isolation of antibodies to targets which cannot be used  
4 for traditional methods of antibody generation due to  
5 toxicity, low immunogenicity or ethical considerations.  
6 In this example we demonstrate the isolation of  
7 specific binding activities against a peptide antigen  
8 from a PDCP library of scFvs from an un-immunised  
9 human.

10  
11 The generation of the library, used for the isolation  
12 of binding activities in this example, is described in  
13 Example 8.

14  
15 Substance P is an eleven amino acid neuropeptide  
16 involved in inflammatory and pain responses *in vivo*. It  
17 has also been implicated in a variety of disorders such  
18 as psoriasis and asthma amongst others (Misery, L.  
19 1997, Br. J. Dermatol., 137: 843-850; Maggi, C. A.  
20 1997, Regul. Pept. 70: 75-90; Choi, D. C. & Kwon, O.J.,  
21 1998, Curr. Opin. Pulm. Med., 4: 16-24). Human  
22 antibodies which neutralise this peptide may therefore  
23 have some therapeutic potential. As this peptide is too  
24 small to coat efficiently on a tube, as described in  
25 Example 3, selection of binding activities was  
26 performed in-solution, using N-terminal biotinylated  
27 substance P and capturing bound PDCP particles on  
28 streptavidin-coated magnetic beads.

29  
30 **Enrichment for substance P binding PDCP particles.**

1 An aliquot of approximately  $10^{13}$  a.r.u. IgM and IgD scFv  
2 library stock was mixed with 1 $\mu$ g biotinylated substance  
3 P in 800 $\mu$ l 4% BSA/0.1% Tween 20/PBS, and allowed to  
4 bind for two hours at ambient temperature. Bound PDCPs  
5 were then captured onto 1ml of BSA blocked streptavidin  
6 coated magnetic beads for 10 minutes at ambient  
7 temperature. The beads were captured to the side of the  
8 tube with a magnet (Promega), and unbound material  
9 discarded. The beads were washed eight times with 1ml  
10 PBS/0.1% Tween 20/ 10 $\mu$ g/ml streptavidin, then two times  
11 with 1ml of PBS by magnetic capture and removal of wash  
12 buffer. After the final wash bound PDCPs were eluted  
13 with 1ml of freshly prepared 0.1M triethylamine for 10  
14 minutes, the beads were captured, and eluted particles  
15 transferred to 0.5ml 1M Tris-HCl pH 7.4. Neutralised  
16 particles were added to 10ml log phase TG1 *E. coli*  
17 bacteria and incubated at 37°C without shaking for 30  
18 minutes, then with shaking at 200rpm for 30 minutes.  
19 10<sup>-3</sup>, 10<sup>-4</sup> & 10<sup>-5</sup> dilutions of the infected culture were  
20 prepared to estimate the number of particles recovered,  
21 and the remainder was spun at 4000 rpm for 10 minutes,  
22 and the pellet resuspended in 300 $\mu$ l 2xTY medium by  
23 vortex mixing. Bacteria were plated onto 2xTY agar  
24 plates supplemented with 1% glucose and 100 $\mu$ g/ml  
25 ampicillin. Colonies were allowed to grow overnight at  
26 30°C. A 100-fold concentrated PDCP stock was prepared  
27 from a 200ml amplified culture of these bacteria as  
28 described above, and 0.5ml used in as second round of  
29 selection with 500ng biotinylated substance P. For this

1 round 100 $\mu$ g/ml streptavidin was included in the wash  
2 buffer.

3

4 **ELISA identification of binding clones.**

5 Binding clones were identified by ELISA of 96  
6 individual PDCP cultures prepared as described in  
7 Example 3 from colonies recovered after the second  
8 round of selection. A Dynatech Immulon 4 ELISA plate  
9 was coated with 200ng/well streptavidin in 100 $\mu$ l /well  
10 PBS for 1 hour at 37°C. The plate was washed  
11 3x200 $\mu$ l/well PBS and incubated with 10ng/well  
12 biotinylated substance P in 100 $\mu$ l /well PBS for 30  
13 minutes at 37°C The plate was washed 3x200 $\mu$ l/well PBS  
14 and blocked for 1 hour at 37°C with 200 $\mu$ l/well 2% Marvel  
15 non-fat milk powder/PBS and then washed 2x200 $\mu$ l/well  
16 PBS. 50 $\mu$ l PDCP culture supernatant was added to each  
17 well containing 50 $\mu$ l/well 4% Marvel/PBS, and allowed to  
18 bind for 1 hour at ambient temperature. The plate was  
19 washed three times with 200 $\mu$ l/well PBS/0.1% Tween 20,  
20 then three times with 200 $\mu$ l/well PBS. Bound PDCPs were  
21 detected with 100 $\mu$ l/well, 1:5000 diluted anti-M13-HRP  
22 conjugate (Pharmacia) in 2% Marvel/PBS for 1 hour at  
23 ambient temperature and the plate washed six times as  
24 above. The plate was developed for 10 minutes at  
25 ambient temperature with 100 $\mu$ l/well freshly prepared  
26 TMB (3,3',5,5'-Tetramethylbenzidine) substrate buffer  
27 (0.005% H<sub>2</sub>O<sub>2</sub>, 0.1mg/ml TMB in 24mM citric acid/52mM  
28 sodium phosphate buffer pH 5.2). The reaction was  
29 stopped with 100 $\mu$ l/well 12.5% H<sub>2</sub>SO<sub>4</sub> and read at 450nm.  
30 Out of 96 clones tested, 10 gave signals greater than  
31 twice background (background = 0.05).

1

2     **Characterization of a binding clone.**

3     A 50-fold concentrated PDCP stock was prepared from a  
4     100ml amplified culture of a single ELISA positive  
5     clone as described above. 10 $\mu$ l per well of this stock  
6     was tested in ELISA as described above for binding to  
7     streptavidin, streptavidin-biotinylated-substance P and  
8     streptavidin-biotinylated-CGRP (N-terminal  
9     biotinylated). Binding was only observed in  
10    streptavidin-biotinylated-substance P coated wells  
11    indicating that binding was specific. In addition,  
12    binding to streptavidin-biotinylated substance P was  
13    completely inhibited by incubating the PDCP with 1 $\mu$ g/ml  
14    free substance P (see Figure 8). The scFv VH (SEQ ID  
15    Nos 15 and 16) and VL (SEQ ID Nos 17 and 18) DNA and  
16    amino acid sequence was determined by DNA sequencing  
17    with oligonucleotides M13REV (SEQ ID No27) and ORSEQFOR  
18    (SEQ ID No 36) and is shown in Figure 9.

19

20    The results indicate that target binding activities can  
21    be isolated from PDCP display libraries of human scFv  
22    fragments.

23

24     **Example 10**

25    In another example the invention provides methods for  
26    screening a DNA library whose members require more than  
27    one chain for activity, as required by, for example,  
28    antibody Fab fragments for ligand binding. To increase  
29    the affinity of an antibody of known heavy and light  
30    chain sequence, libraries of unknown light chains  
31    co-expressed with a known heavy chain are screened for

1 higher affinity antibodies. The known heavy chain  
2 antibody DNA sequence is joined to a nucleotide  
3 sequence encoding an estrogen receptor DNA binding  
4 domain in a phage vector which does not contain the  
5 estrogen receptor HRE sequence. The antibody DNA  
6 sequence for the known heavy chain (VH and CH1) gene is  
7 inserted in the 5' region of the estrogen receptor DBD  
8 DNA, behind an appropriate promoter and translation  
9 sequences and a sequence encoding a signal peptide  
10 leader directing transport of the downstream fusion  
11 protein to the periplasmic space. The library of  
12 unknown light chains (VL and CL) is expressed  
13 separately from a phagemid expression vector which also  
14 contains the estrogen receptor HRE sequence. Thus when  
15 both heavy and light chains are expressed in the same  
16 host cell, following infection with the phage  
17 containing the heavy chain-DBD fusion, the light chain  
18 phagemid vector is preferentially packaged into mature  
19 phage particles as single stranded DNA, which is bound  
20 by the heavy chain-DBD fusion protein during the  
21 packaging process. The light chain proteins are  
22 transported to the periplasm where they assemble with  
23 the heavy chain that is fused to the DBD protein as it  
24 exits the cell on the PDCP. In this example the DBD  
25 fusion protein and the HRE DNA sequences are not  
26 encoded on the same vector, the unknown peptide  
27 sequences are present on the same vector as the HRE  
28 sequence. Peptide display carrier packages (PDCP) which  
29 encode the protein of interest can then be selected by  
30 means of a ligand specific for the antibody.

Table 1 (i) Oligonucleotide primers used for human scFv library construction

## cDNA synthesis primers

IgMCDNAFOR	TGGAAGAGGCACGTTCTTCTTT
IgDCDNAFOR	CTCCTTCTTACTCTTGCTGGCGGT
IgκCDNAFOR	AGACTCTCCCCTGTTGAAGCTCTT
IgλCDNAFOR	TGAAGATTCTGTAGGGGCCACTGTCTT

## JHFOR primers

JH1-2FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCAGGGTGCC
JH3FOR	TGAACCGCCTCCACCTGAAGAGACGGTGACCATTGTCCC
JH4-5FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCAGGGTTCC
JH6FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCGTGGTCCC

## VH family BAK primers

VH1BAK	TTTTTGGCCCAGCCGGCCATGGCCCATGGCCAGGTGCAGCTGGTGCAAGTCTGG
VH2BAK	TTTTTGGCCCAGCCGGCCATGGCCCATGGCCAGGTCAACTAAGGGAGTCTGG
VH3BAK	TTTTTGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGAGTCTGG
VH4BAK	TTTTTGGCCCAGCCGGCCATGGCCCATGGCCAGGTGCAGCTGCAGGAGTCGGG
VH5BAK	TTTTTGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGTTGCAGTCTGC
VH6BAK	TTTTTGGCCCAGCCGGCCATGGCCCATGGCCAGGTACAGCTGCAGCAGTCAGG

## Light chain FOR primers

SCFVKFOR	TTATT CGCGGCCGCCTAACACAGAGGCAGTTCCAGATTTC
SCFVλFOR	GTC ACTT CGCGGCCGCCTACAGTGTGGCCTTGTGGCTTG

## VK family BAK primers

VK1BAK	TCTGGCGGTGGCGGATCGGACATCCAGATGACCCAGTCTCC
VK2BAK	TCTGGCGGTGGCGGATCGGATGTTGTGACTCAGTCTCC
VK3BAK	TCTGGCGGTGGCGGATCGGAAATTGTGTTGACGCAGTCTCC
VK4BAK	TCTGGCGGTGGCGGATCGGACATCGTGATGACCCAGTCTCC
VK5BAK	TCTGGCGGTGGCGGATCGGAAACGACACTCACGCAGTCTCC
VK6BAK	TCTGGCGGTGGCGGATCGGAAATTGTGCTGACTCAGTCTCC

## JK FOR primers

JK1FOR	TTCTCGTGC GGCGC CT AAC GTT GAT TT CC AC CT TG GT CCC
JK2FOR	TTCTCGTGC GGCGC CT AAC GTT GAT CT CC AG CT TG GT CCC
JK3FOR	TTCTCGTGC GGCGC CT AAC GTT GAT AT CC ACT TT GG T CCC
JK4FOR	TTCTCGTGC GGCGC CT AAC GTT GAT CT CC AC CT TG GT CCC
JK5FOR	TTCTCGTGC GGCGC CT AAC GTT A AT CT CC AG CT TG GT CCC

V $\lambda$  family BAK primers

V $\lambda$ 1BAK	TCTGGCGGTGGCGGATCGCAGTCTGTGTTGACGCAGCCGCC
V $\lambda$ 2BAK	TCTGGCGGTGGCGGATCGCAGTCTGCCCTGACTCAGCCTGC

Table 1 (ii) Oligonucleotide primers used for human scFv library construction

V $\lambda$ 3aBAK	TCTGGCGGTGGCGGATCGTCCTATGTGCTGACTCAGCCACC
V $\lambda$ 3bBAK	TCTGGCGGTGGCGGATCGTCTTGAGCTGACTCAGGACCC
V $\lambda$ 4BAK	TCTGGCGGTGGCGGATCGCACGTTACTGACTCAACCGCC
V $\lambda$ 5BAK	TCTGGCGGTGGCGGATCGCAGGCTGTGCTCACTCAGCCGTC
V $\lambda$ 6BAK	TCTGGCGGTGGCGGATCGAATTATGCTGACTCAGCCCCA

J $\lambda$  primers

J $\lambda$ 1FOR	TTCTCGTGC GGCGC CT AAC C T A G G A C G G T G A C C T T G G T C C C
J $\lambda$ 2-3FOR	TTCTCGTGC GGCGC CT AAC C T A G G A C G G T C A G C T T G G T C C C

Jλ4-5FOR TTCTCGTGC GGCCGCCTAACCTAAAACGGTGAGCTGGGTCCC

Linker primers

LINKAMP3 CGATCCGCCACCGCCAGA

LINKAMP5 GTCTCCTCAGGTGGAGGC

LINKAMP3T CGATCCGCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGAC

69

1